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Title:
Interaction between immune cells phenotype and the microbiome in B cells immunodeficiency diseases
miRNA & microbiomes in PIDDs

Interaction between immune cells phenotype and the microbiome in B cells immunodeficiency diseases:

miRNA & microbiomes in PIDDs

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Declaration

I declare that the work presented in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author. In addition, I confirm that the hardcopy and the e-submission are identical."

SIGNED: Maryam Ali Y Al-Nesf Al-Mansouri

DATE: 07/08/2019

Abstract

Background:
Deficiency in B-cell development is the most frequent of primary immunodeficiency disorders (PIDD), characterised by complications, making diagnoses and treatment challenging. Patients receive intravenous immunoglobulin (IGIV) and micronutrient supplementation to improve prognosis, with likely changes in biomarkers. Associations were sought between medico-clinical parameters, serum exosomal miRNA and the gut microbiome in patients with PIDD after intravenous immunoglobulin (IGIV) and micronutrient supplementation.

Methodology:
Ethical approval was granted. Retrospective analysis of data from PIDD (n=48), which included phenotypic classification, inflammatory markers, age at presentation and vitamin D levels, on a subset (n=27). Intervention studies (n=10) of serum exosomal microRNA expression and the gut microbiome were done pre-/post-IGIV and -vitamin (A, E, B, and D) supplementation.

Results:
Vitamin D deficiency was apparent in patients, compared to reference ranges. Comparisons between patients with PIDD with no complications, as opposed to those with bronchiectasis and/or allergy, showed comparable vitamin D levels ($p>0.05$). Elevated CRP and lower IgG levels were apparent in patients with bronchiectasis and allergy complication ($p<0.05$). White blood cell (WBC) counts were higher in patients without complications ($p=0.03$), perhaps indicating a lower infection rate in this group. Bronchiectasis was higher in patients diagnosed at paediatric age (<14 years; 11 out of 12; 91.7%) compared to adults (9 out of 24; 37.5%) ($p=0.002$). Smoking was not associated with an increased rate of complications, but males appeared more prone to bronchiectasis ($p=0.003$).
Exosomal miR showed up-regulation in 2 miRNAs (hsa-miR-122-5p and miR-4497) following IGIV and vitamin supplementation. No changes in microbiome diversity were seen.

Conclusion:
Elevated CRP is a biomarker for B-cell immunodeficiency and bronchiectasis, with miRs, 122-5p and 4497, as mediators of both IGIV and vitamin supplementation. The robustness of CRP and miRs as biomarkers and their role in PIDD need to be confirmed in larger cohorts. Understanding of aetiology of the disease could help design effective therapies and monitoring methods.

Keywords: B cell immunodeficiency, primary immunodeficiency, hypogammaglobulinaemia

Dedication and Acknowledgements

I am indebted to many people who helped me throughout this research project.

Firstly, I'm thankful for my supervisor, Dr Vidya Mohamed-Ali, for her continuous support and help during all the steps in order to carry out this project. Her unlimited guidance allowed me to progress positively in the research field. Her experience, which was shared with patience, helped to develop a good understanding and passion for research and science. I thank my supervisor Dr David Morgan for his advice and guidance in the ethics application, patient recruitment, and with further discussions regarding formulating a proper hypothesis, experimental design, and the results. I also thank both, my supervisor, Dr Wael Kafienah, for his continuous support and advice throughout my study, and Dr Mohammed Al-Maadheed, who recognised the need for this program in Qatar and works hard to make this dream become true and facilitate the process to help us as junior scientists become involved in such a program. Thanks to Dr Halima Bensmail and Dr Mostafa for their help in microbiome and miRNA analyses, and to the statistician Dr Prem Chandra for his help in analysing the statistics related to patients' characteristics.

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Abbreviations

PIDD:	Primary immunodeficiency disease
PID:	Primary immunodeficiencies
CVID:	Common Variable Immunodeficiency
PAD:	Predominantly Antibody Deficiency
SAD:	Specific/selective Antibody deficiency
SPAD:	Specific polysaccharide antibody deficiency
SCID:	Severe combined immunodeficiency
IUIS:	International Union of Immunological Societies phenotypic classification
IEI:	Inborn Error of Immunity
ESID:	European Society for Immunodeficiencies
OMIM:	Online Mendelian Inheritance in Man
CFB:	Complement factor B (protective serum molecules from the complement system)
C3:	Complement 3 (protective serum molecules from the complement system)
CARD11:	Caspase recruitment domain-containing protein 11
STAT1:	Signal transducer and activator of transcription 1
STAT3:	Signal transducer and activator of transcription 3
WAS:	Wiskott-Aldrich Syndrome Protein Coding Gene.
JAK1:	Janus Kinase 1
IFIH1:	Interferon Induced with Helicase C Domain 1 (Protein Coding)
ZAP70:	Zeta chain of T Cell Receptor Associated Protein Kinase 70 (Protein Coding)
CRP:	C-reactive protein
RNA:	Ribonucleic acid
miRNA:	microRNA
RIN:	RNA Integrity Number
NK:	Natural Killer cells
TNF:	Tumour necrosis factor (a signalling protein or cytokine)
LPS:	Lipopolysaccharide
IL:	Interleukin
IGIV:	Intravenous Immunoglobulins
IQR:	Inter-Quartile Range
SD:	Standard Deviation

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Publications

Conference proceedings

1. Al-Nesf MA, Morgan D, Chandra P, Mohamed-Ali V. Stratification of Patients with Primary Immunodeficiency Diseases: Relevance to Etiology and Therapy. 5th Annual Junior Symposium at ADLQ (JS ADLQ) 19th Dec 2017.
2. Al-Nesf MA, Morgan D, Chandra P, Mohamed-Ali V. Stratification of Patients with Primary Immunodeficiency Diseases. Oral presentation at the Immunology 2018 Conference (with theme “Spreading the new trends in Immunology”), July 05-07, 2018 Vienna, Austria.
3. Al-Nesf MA, Morgan D, Chandra P, Mohamed-Ali V. Identification of Biomarkers associated with immunoglobulin therapy and dietary vitamin supplementation in patients with B cells immunodeficiency diseases. 6th Annual Junior Symposium at ADLQ (JS ADLQ) 11th Dec 2018.

CHAPTER 1

INTRODUCTION

1.1. Primary Immunodeficiency Diseases (PIDD)

1.1.1. Definition and background

Primary immunodeficiency diseases (PIDD) are a group of heterogeneous inherited disorders of the immune system[1-4], characterised by poor or absent function in one or more components of the immune system[5-6]. Both the acquired form and the inherited subclasses of PIDD that give rise to defects in immune system development and function have been described[7]. PIDD differs from secondary immunodeficiency that develops because of haematological malignancies or certain immunosuppression medication that is used following transplantation, for systemic autoimmune disease, and as part of cancer chemotherapy[1,8]. The classical understanding of primary immunodeficiency disorders is as abnormalities in the development and maturation of cells of the immune system. This defect results in increased susceptibility to infection. For example, recurrent pyogenic infections occur with defects of humoral immunity and correspond roughly to defects in one of the two principal types of immunocompetent cells, B lymphocytes, and opportunistic infections with defects in cell-mediated immunity that correspond to T lymphocytes defects, and, therefore, to cellular immunity[9]. However, the clinical manifestations of PIDDs are highly variable[4], and patients with PIDD may present with increased susceptibility to infection, immune dysregulation (autoimmune disease and aberrant inflammatory responses), and malignancy[1].

Currently, the term primary immunodeficiency is considered a limitation to the wide spectrum of the disease and depicts only the susceptibility to infection as the main manifestation. Advances in the understanding of the broader meaning of PIDD have led to the inclusion of i*mmune dysregulation* diseases, auto-inflammatory disorders, and interferonopathies. The use of the phrase ‘inborn errors of immunity’ was utilised as a descriptive term in the 2017 updated classification of the disease entities by the International Union of Immunological Societies (IUIS)[2].

1.1.2. Description and classification

PIDDs are classified into nine distinct categories based on a combination of mechanistic and clinical descriptive characteristics by IUIS 2017 (Table 1). These categories are further divided into disease sub-categories according to common phenotypes. However, despite such phenotypic classification, it should not be assumed that the presentation of those immunodeficiency disorders is homogeneous. A few points of importance in the 2017 updated classification are that the mode of inheritance is not one of the classification criteria, and any defective gene can be the cause in more than one phenotype. To date, nine genes have been reported: CFB, C3, CARD11, STAT1,

STAT3, WAS, JAK1, IFIH1, and ZAP70, representing both loss-of-function and gain-of-function variants[2].

IUIS Classification and Division into Disease Groups
1. Combined T- and B-cell immunodeficiency
2. Combined immunodeficiencies with associated or syndromic features
3. Predominantly antibody deficiencies
4. Diseases of immune dysregulation
5. Congenital defects in phagocyte number and/or function
6. Defects in natural immunity (innate immunity)
7. Auto-inflammatory disorders
8. Complement deficiencies
9. Phenocopies of inborn errors of immunity.

Table 1: The 2017 IUIS phenotypic classification for primary immunodeficiencies.

Nine subcategories of Primary Immunodeficiency were established by the Inborn Errors of Immunity Committee (IEI), which consists of experts in primary immunodeficiencies and contribute to the biennial IEI classification reports under the umbrella of the International Union of Immunological Societies. (*Note: adapted from Picard et al. 2018* [2] *and Bousfiha et al. 2018* [3]).

1.1.3. Epidemiology: Prevalence, Age, Sex, and Ethnicity

PIDD is not a common disorder, and few countries have established a registry to improve the reliability and quality of the epidemiological data. However, many do not have available information, and even those with registries are facing under-reporting, a lack of awareness, and under-diagnosis; factors which, in combination, lead to the underestimation of prevalence[10]. Since February 2017, 354 PIDD have been established by the Inborn Errors of Immunity (IEI) committee. These disorders were categorised with 344 different gene defects and a wide spectrum of phenotypes[2].

National, transnational and international registries are identifying missing data about these diseases (Figure 1). The currently available international registries aim to predict the prevalence, rate and different types of these rare disorders[11-12]. The increase in physicians' awareness and recognition allowed its reported prevalence to increase from 38.9 to 50.5 per 100,000 among the privately insured, and from 29.1 to 41.1 per 100,000 publicly insured persons, from administrative healthcare databases between 2001 and 2007 in the USA. Moreover, it was found that B-cell defects predominated and that the prevalence was more than double in Caucasian Americans (Whites), compared with African Americans or Hispanics[11-13]. This figure is comparable with the prevalence of 1 in 2000 to 1 in 1200 live births estimated in the USA[2,14]. While most forms of PIDD are rare, with incidences of around 1 in 500,000 of the population, some specific PIDD are relatively common. One of these immunodeficiencies is IgA deficiency, which accounts for 1 in 500 in Caucasian populations, yet only accounts for 1 in 18,000 in East Asians[12]. However, much is dependent on the diagnostic skills and medical resources available in different countries[15].

The prevalence of PIDD from the published literature and different countries' registries has received particular attention internationally[10-28]. The ESID, for example, went online in 2004 and is one of the most important and popular resources for researchers and physicians interested in immunodeficiency. As of January 2019, predominantly antibody disorders account for 10,612 of cases (50.4%), other well defined PIDs account for 2967 (14.1%), and combined immunodeficiencies account for 2041 (9.7%)[16,19-21]. European countries including Italy, France, and Spain initially had their own registries, but subsequently adopted the ESID platform[10-13,19-21]. The National United Kingdom Primary Immunodeficiency (UKPID) Registry adopted the ESID platform in 2005. The second published report from the UKPID indicated a doubling of PIDD (from 2229 to 4758 patients) with a minimum PID prevalence of 5.90/100,000 and an average incidence

of 7.6 cases per 100 000 UK live births between 1980 and 2000. The report also showed that females accounted for 2399, those aged 16 years or less for 807 (17%), there was a molecular diagnosis in 1035 patients (21.8%), consanguinity in 118 of 4097 patients (2.9%) and familial cases accounted for 3971 (24.4%). The antibody disorders dominate, with 2821 (59.7%), of which CVID is the most frequently reported PID (1404; 29.7%)[22]. The US Immunodeficiency Network (USIDNET) Registry began in 1992 and reported 2622 predominantly antibody disorders among 5204 participants[24]. The Latin American Group for PIDD registry is one of the oldest registries (established in 1993) to have adopted the ESID platform. PAD represented 53.2% of the 3321 registered PIDD[25]. The Australasian Society of Clinical Immunology and Allergy Registry (ASCIA PID) Register in Australia and New Zealand was launched in 2003. They reported 1209 cases (70% with PAD), with an estimate of the true prevalence of 13.2–14.5/100,000[26]. The Iranian Primary Immunodeficiencies Registry is one of the geographically closest registries to Qatar. Over 30 years of experience in PIDD, they reported 3056 (1204 females) from 31 medical centres. The predominantly antibody deficiency was the most common, with 903 cases (29.5%). The genetic defect was identified in 1014 patients (33.1%); 79.3% were autosomal recessive and combined immunodeficiencies, with associated or syndromic features accounting for 529. The cumulative incidence of PID in Iran during the past ten years was estimated to be around 81 cases per 1,000,000 inhabitants[27]. In the Middle Eastern region consanguinity is high, around 20-56% as opposed to 1-9% globally, and contributes significantly to genetic disorders such as PIDD. Countries in this region, including: Morocco, Tunisia, Israel, Kuwait, Iran Egypt, Turkey, Saudi Arabia, Qatar, and Oman, have reported PIDD from their national registry, survey or referral centre. (Figure 1c [28]).

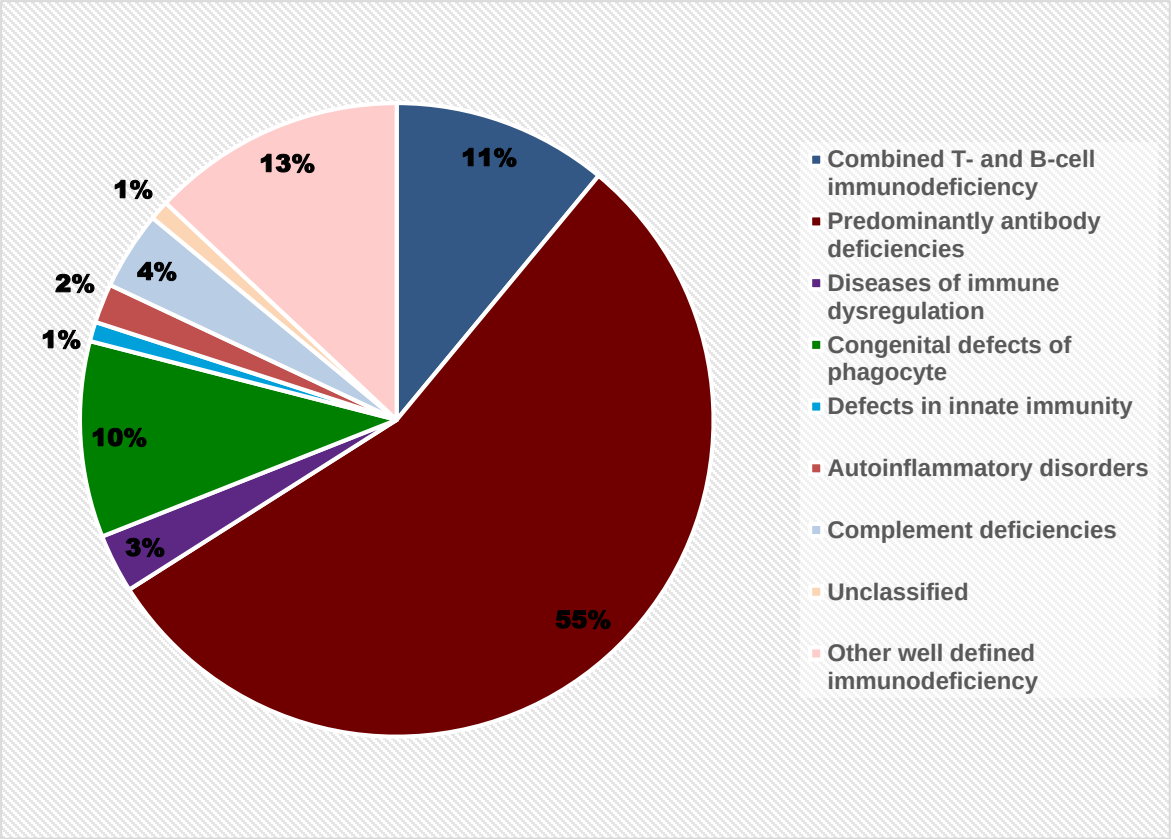
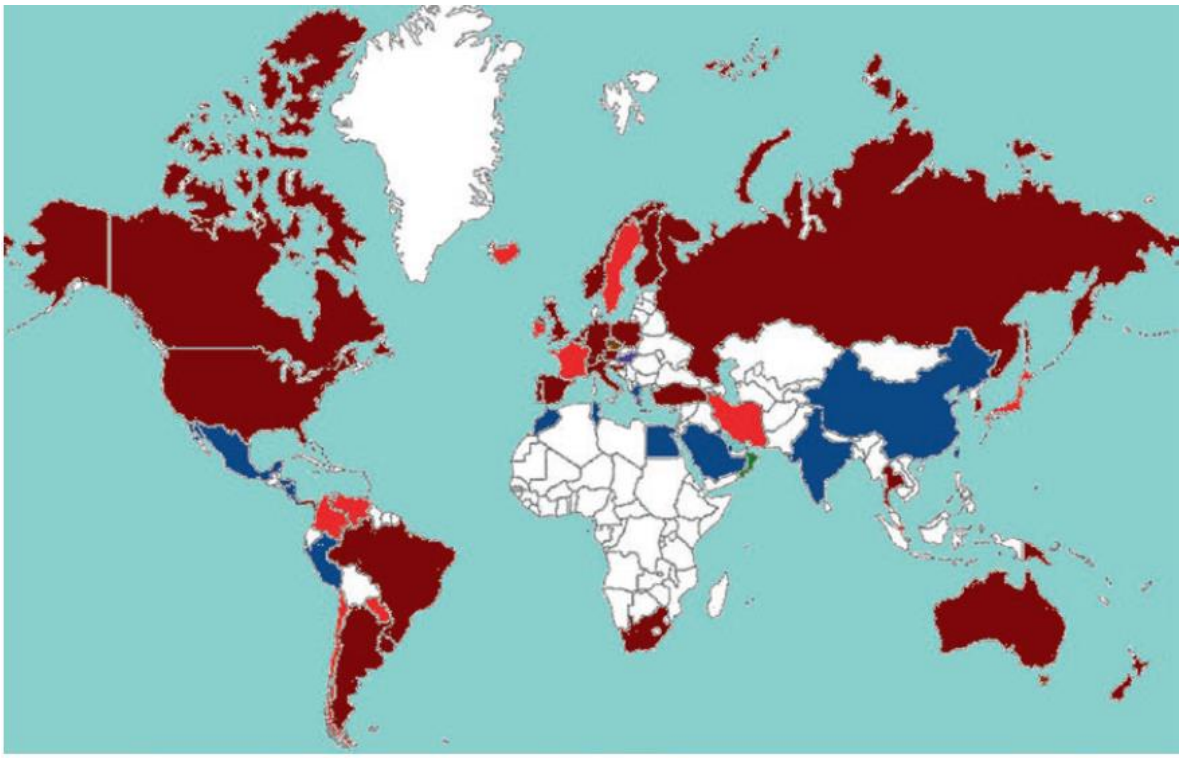


Figure 1a: The relative frequencies of the various PIDDs.



- Predominantly antibody deficiencies (>50%).
- Predominantly antibody deficiencies (<50%).
- Combined T- and B-cell and other well-defined PIDDs.
- Congenital defects of phagocytes.
- Complement deficiencies.

Figure 1b: Global prevalence.

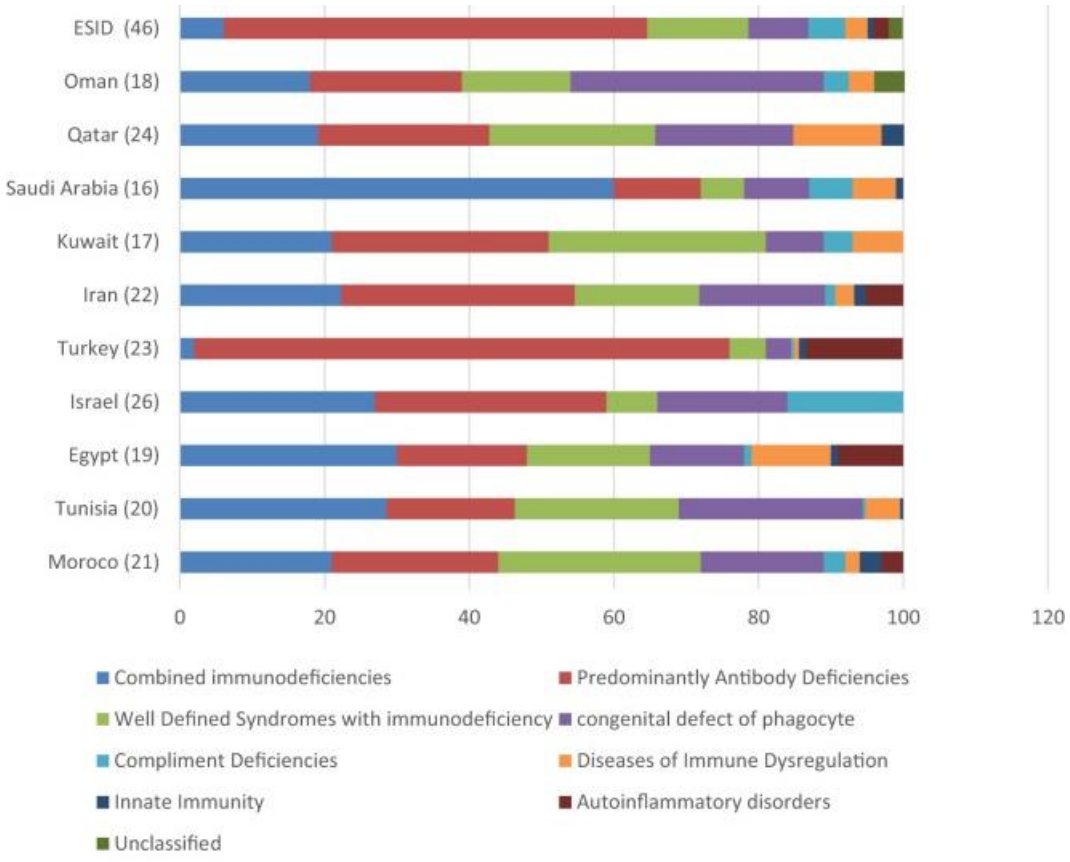


Figure 1c: PIDD distribution in the MENA region.

Figure 1.0: Relative Frequency and Prevalence of PIDDs (Global & MENA).

1a: Relative frequencies of primary immunodeficiency diseases as described by Rezaei *et al*. The pie chart showed that >50% represent cases with predominantly antibody deficiencies across the world.

1b: Distribution of different types of primary immunodeficiency diseases in the world. The predominantly antibody deficiency prevalence varies across the globe, and we can see that the disease prevalence is less than 50% in some countries.

1c: The Middle East and North Africa (MENA) countries have higher consanguinity than other countries in the globe (reaching up to 56%). Hence, the rate of PIDD is expected to be higher. The chart showed the frequencies in the MENA region in comparison to the European prevalence. While antibody deficiency is predominant, some Arab countries showed a lower prevalence with a higher prevalence of combined immunodeficiency, namely KSA, Egypt, and Tunisia. (*Note: adapted from Rezaei et al. 2017* [12] *and Al-Mousa et al. 2017* [28]).

1.1.4. Clinical evaluation and management of PIDD

Whilst evaluating immunodeficiency, it is important, where possible, to document the origin and type of infections, the causative organisms, and the treatment course and response. Such evaluation is necessary to detect other non-infectious conditions, such as allergy, or to distinguish viral from bacterial infections. Anatomical defects, allergy, and metabolic disorders should be considered during different PIDD evaluation and attention paid to some diagnostic hints, such as, the presence of food and inhaled allergic diseases. In general, from a clinical perspective, taking proper history and following up the clinical presentation aids in the diagnosis and management. Using simple screening tests such as complete blood count followed by advanced tests is the usual approcah[1-2]. Serious morbidity and early mortality may be prevented by accurate and early diagnosis and a stepwise approach that includes a clinical and immunological evaluation with the help of the international diagnostic protocols designed by the European Society for Immunodeficiencies (ESID)[29], followed by flow cytometric immunophenotyping and functional studies. This approach guides the selection of candidate genes for molecular diagnostics.

The genetic defect has not yet been identified in all PIDD patients. Moreover, the diversity of the clinical presentation of PIDD with genetic defects in known candidate genes are increasing, which necessitates more extensive diagnostic testing in specific cases[30-31]. Two common causes are to be considered when evaluating immune-related conditions. The first is HIV infection. It is one of the ten leading causes of death in the USA among the age groups from 15-65[32]. HIV testing is mandatory when evaluating cases of PIDD[33]. The second is nutritional status, specifically, malnutrition. Nutrition is one of many factors that may affect the immune system, and malnutrition does not just mean energy and protein deficiency. Macronutrients (fat, carbohydrate, protein), as well as micronutrients deficiency (vitamins, minerals, water), are harmful to immune functions. Balanced nutrition helps in the body's protection against infection and supports immune function[34-35]. Immuno-nutrition is a term used to describe special diets believed to augment the immune system. In meta-analyses, the benefit of immune-nutrition was pronounced in surgically ill patients[36-37].

Some attention has been focused on the use of multiple micronutrients as a means of improving the immune system, including essential amino acids, the essential fatty acid linoleic acid, vitamin A, folic acid, vitamin B6, vitamin B12, vitamin C, vitamin E, Zn, Cu, Fe, and Se[38].

Multiple studies have also been conducted to evaluate the effect of diet at a cellular level. One study, in which the effect of exercise and two unbalanced diets was evaluated in mouse models, revealed that changes in leptin and adiponectin levels were associated with the increases in total lymphocytes, CD4+ cells, and CD8+ cells. This was also associated with a reduction in B-cells. These diets were found to play a role in the induction of glycaemia and oxidative stress that modify the proliferation of total lymphocytes and the percentage of B-cells, through alterations in proteins by carbonylation. This phenomenon was not altered by moderate exercise[39]. In another study, a potential health benefit was suggested by consuming fruit and vegetable juice powder concentrate capsules that leads to the suppression of peripheral blood lymphocyte (PBL) DNA damage[40]. The effects of other micronutrients (vitamins A and D) on the immune system were also studied[34].

1.2. Predominant antibody deficiency (common variable immunodeficiency, CVID, and specific antibody deficiency, SAD)

Predominant or primary antibody deficiencies (PAD) is the second category under 2017 IUIS classification[2]. It requires lifelong replacement therapy with immunoglobulin (Ig)G to reduce the incidence and severity of infections[41]. Diseases under this category include X-linked agammaglobulinaemia, X-linked hyper-IgM, severe combined immunodeficiency, Wiskott-Aldrich syndrome, selective IgG class deficiency with specific antibodies deficiency, and common variable immunodeficiency (CVID), among others[2]. PAD causes recurrent bacterial infection affecting multiple systems, including the gastrointestinal and respiratory tracts. Affected individuals have changes in B-cell function, reduced levels of naïve T cells and Treg cells, an increased risk of malignancy, chronic immune activation, altered levels of cytokine production, and autoimmunity[7]. PAD might depend on a variety of defects that interfere with B-cell development, maturation, and/or function. PAD form the largest category, followed by combined B- and T-cell deficiencies[2,42]. The subclass SIgAD is the most common of all PIDD, yet most patients with SIgAD are asymptomatic.

In contrast, CVID is the commonest clinically relevant primary immunodeficiency[15]. The different B-cell deficiency diseases are directly related to the stage at which B-cell development and arrest happened[6,43-46] (Figure 2).

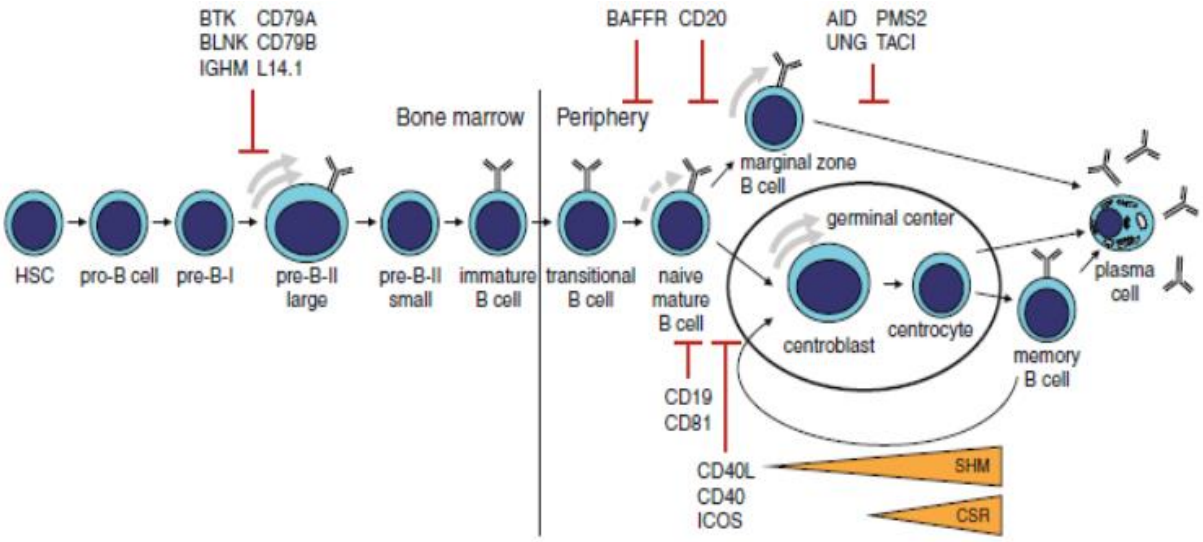


Figure 2: The effect of the monogenic defect on the B-cell development in PIDDs.

The following primary antibody deficiency diseases (X-linked agammaglobulinaemia (XLA), selective IgA deficiency (SIgAD), Common Variable Immunodeficiency (CVID), and Hyper IgM syndrome (HIGM) have defects or arrest in B-cell developmental different stages and can be explained using examples of monogenic defects. The genetic defects at different locations lead to different antibody deficiency diseases. In X-Linked agammaglobulinaemia (BTK gene), the B-cell arrest occurs at the preB-cell, i.e. early in B-cell development); a process happens in the bone marrow (antigen-independent pathway) and results in the absence of B-cells, leading to an absence of Igs. In contrast, in the periphery (via the antigen-dependent pathway), the CVID, SIgAD, and HIGM develop due to B-cells being unable to differentiate into various different Ig isotype-producing cells, thereby resulting in variable degrees of Ig deficiency. In hyper-IgM syndrome (HIGM), defects in switching from IgM isotype production into the production of other isotypes resulted in high levels of IgM and low levels of other Igs (*Note: adapted from Driessen et al. 2011* [46]).

1.2.1. Common Variable Immunodeficiency

Common variable immunodeficiency (CVID; OMIM*240500), represents a heterogeneous group of syndromes characterised by low levels of immunoglobulins and impaired antibody response to both polysaccharide and protein antigens. Moreover, patients have increased susceptibility to autoimmunity and malignancy[15,47]. The involvement of different organs is observed in patients with CVID (Figure 3). Fewer than 10% of CVID have a definitive molecular genetic diagnosis; similarly, fewer than 10% have a positive family history[15,47].

According to data from the ESID registry, 30% of the patients had CVID. Both sexes are affected equally. The prevalence is 1 in 50,000 to 1 in 200,000, with a reported incidence of 1 in 75,000 live births. The disease is sporadic in distribution, with up-to 25% having a family history, typically with autosomal dominant inheritance. The disease can present in mid-childhood to mid-adulthood; however, it can be variable. These data give an overview of the age at presentation of CVID and shows that it has a bimodal distribution[14]. The largest series of CVID by Cunningham-Rundles *et al* included 248 patients aged 3–79 years with an average delay of diagnosis from the onset of symptoms of around 5-6 years in male and female respectively[47]. Regional differences are often an issue in genetic disorders, and depend on many factors. Asians and Afro-Americans have lower rate of CVID. The disease can occur at any age and it seems that both male and female have similar incidence of development of the disease[11].

Interestingly, in Finland, CVID was found to be more prevalent than expected[48], likely due to the genetic isolation and potential founder effects in the Finnish population. Also, this can be attributed to the progressively improved diagnosis and awareness in the primary health care sector.

1.2.2. Selective IgA, IgG2 subclass, and specific anti-polysaccharide antibody deficiency

In IgA and IgG subclass deficiency, terminal differentiation of B-cells fails. SIgAD is the most common immunodeficiency, with 1 in 500 Caucasians having the defect. However, it is not common in other ethnic groups, such as in Asians. People with SIgAD have tendency for immune-complex diseases (Type III hypersensitivity) and allergies. Also, recurrent infection with encapsulated organisms is frequent in up to 20% of IgA-deficient individuals due to concomitant lack IgG2 and IgG4 immunoglobulin subclasses. A deficiency of IgG2 alone is a predisposing factor to recurrent infections because most antibodies to the capsular polysaccharides of pyogenic bacteria are of the IgG2 subclass. These class and subclass deficiencies result from a failure in the terminal differentiation of B-cells. The post-pneumococcal polysaccharide vaccination

response is one of the tests used in the clinical immunology practice to evaluate patients with PAD[46] (Table 2).

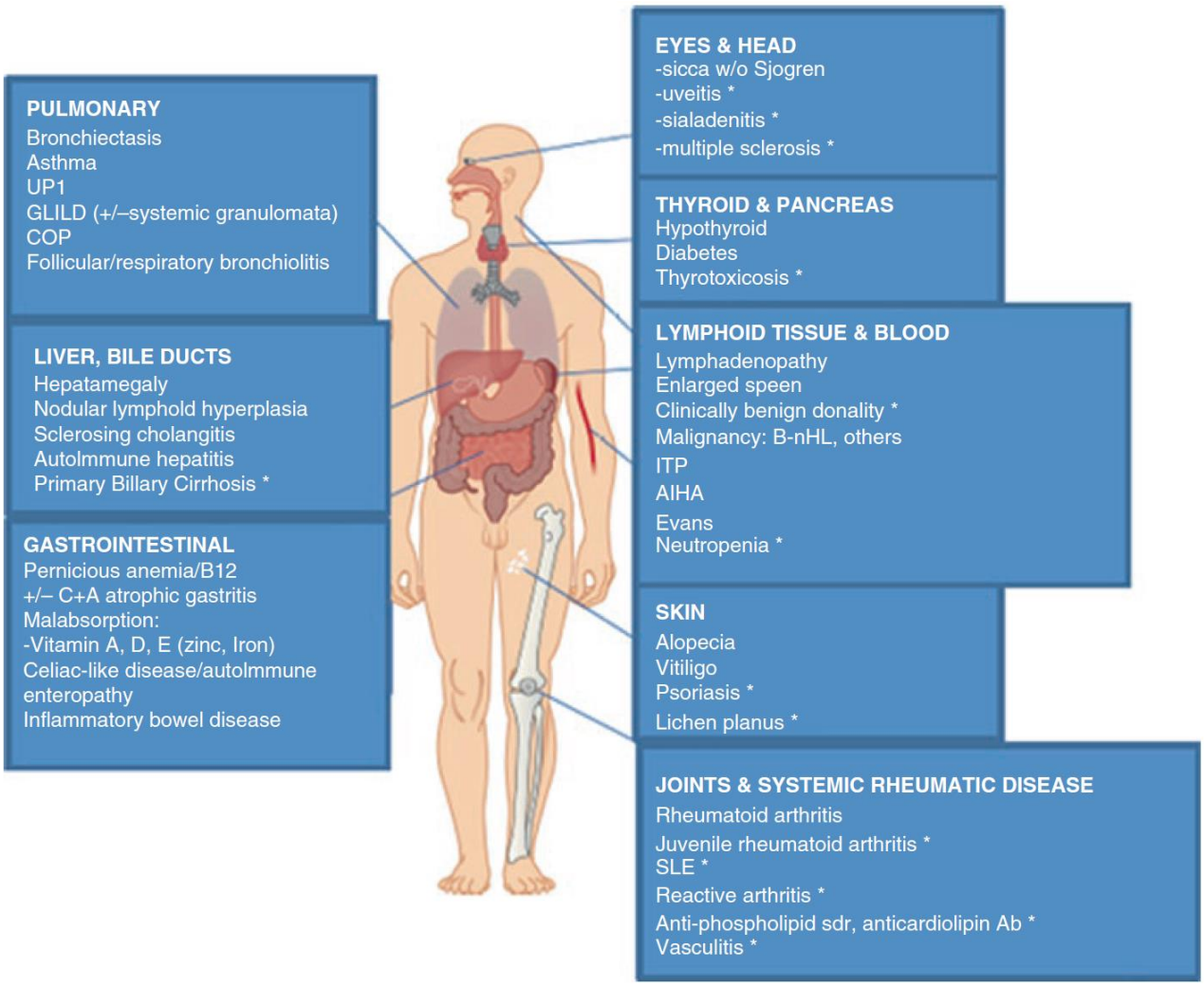


Figure 3: CVID can affect multiple organs and systems in the human body.

Immune disturbances in common variable immunodeficiency resulted in recurrent infections, autoimmune, bronchiectasis, and lymphoproliferative complications (*Note: adapted from Rezaei et al. 2017* [12]*).*

Pneumococcal Serotypes	Conjugate Vaccine			Antibodies level by ELISA (mg/L)			Antibodies level by multiplex bead assay (mg/L)
	PCV-7	PCV-10	PCV-13	3-15 yrs.	19-30 yrs.	3-30 yrs.	3-30 yrs.
1		X	X				0.53
3			X	0.99	0.58	0.67	0.64
4	X	X	X	0.73	0.37	0.45	0.49
6B	X	X	X				0.8
7F		X	X				1.45
8							1.02
9N				0.78	0.23	0.46	0.63
9V	X	X	X				0.69
12F							0.46
14	X	X	X				0.57
18C	X	X	X	0.55	0.31	0.31	0.34
19A			X				0.96
19F	X	X	X	1.66	0.91	1.04	0.74
23F	X	X	X				0.25

Table 2: PIDD assessment by evaluating post-vaccination response (measuring pneumococcal polysaccharide serotypes before and after the vaccine).

An important part of the evaluation of humoral Immunodeficiency is done through measuring post pneumococcal vaccine response (by measuring serum specific serotypes before and 2-6 weeks following vaccination). The common pneumococcal serotypes used in clinical practice to evaluate cases with PAD are listed above. The cut-off value for each serotype, based on different laboratory assays, (ELISA vs. multiplex beads) is compared (*Note: adapted from Driessen et al. 20111* [46]).

1.3 Potential mediators of PIDD

Multiple factors, including Immunoglobulin IGIV administration, microbiome composition, and the deficiency of various micronutrients, have been assessed as potential mediators of CVID[49-57]. While micronutrients were observed to induce particular disease phenotypes[49-51], the different cytokines (IL-10, IL21, and IL-4) have also received attention and been evaluated in different clinical phenotypes of CVID patients[52]. Microbiome and microbial dysbiosis play meticulous roles in immune homeostasis and during inflammatory response. However, there are currently very few studies that suggest unifying or multiple mechanisms for the observed clinical outcome[53-57]. The various factors that are set out below merit further investigation.

1.3.1. Immunoglobulin replacement therapy has an immunomodulatory effect on PIDD

IGIV has proven to be an effective treatment for many diseases, including PIDD. It works as anti-inflammatory and immunomodulator for multiple autoimmune diseases and is given in high doses for this purpose. For PIDD, IGIV is administered at lower concentrations as a replacement therapy. Although the full immunomodulatory mechanism(s) are not fully understood, multiple mechanisms have been postulated in the literature[58-60] (Figure 4).

Immunomodulatory Actions of Intravenous Immunoglobulin

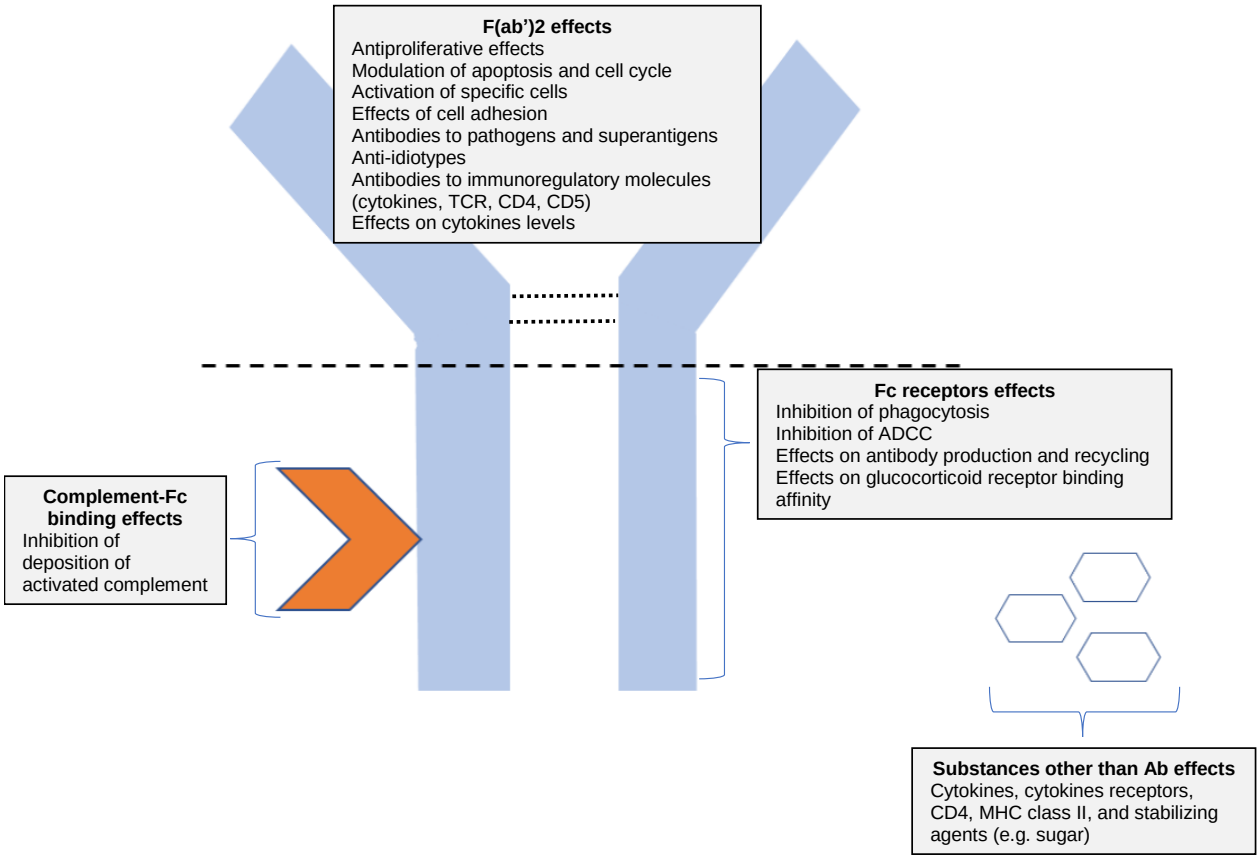


Figure 4: components of IGIV that exerts function.

Four components in the IGIV preparation summarized by *Sewell et al.*: (1) the variable regions $F(ab')_2$, (2) Fc on Fc receptors (FcR), (3) complement binding within the Fc fragment, and (4) other immunomodulatory substances other than antibody in the IGIV preparations. (TCR, T-cell receptor; ADCC, antibody-dependent cellular cytotoxicity) (*Note: adapted from Sewell et al. 2002* [60]).

1.3.2. Micronutrients play a recognized role

The nutritional status of an individual has been shown to have a significant effect on the immune system, both at the macro- and micronutrient levels. There has been long-standing interest in the effect of micronutrients, as they can be used in low doses to affect a change. The vitamins have been investigated extensively in the immune *milieu*. Vitamins play an important role in growth and development, with important neurologic and digestion functions, by supporting the immune system. Moreover, they also maintain normal cellular function in general. Vitamin deficiency depresses the production and causes dysfunction of immune cells. It is well documented that vitamins are considered important in metabolism and are effective in provoking resistance and inducing immunity. Vitamin A, vitamin C, pyridoxine, pantothenic acid, and thiamine have a role in fighting infections, with roles in immunity also reported for B and D vitamins[61].

Vitamin B6

Vitamin B6 deficiency causes skin, nerve, and coronary artery disease. The deficiency of vitamin B6 is mainly associated with malnutrition, malabsorption, and immunodeficiency. In patients with CVID, plasma vitamin B6 levels were found to be reduced. In peripheral blood, the reduced vitamin B6 was not found to be associated with CD4, CD8, CD16, and $CD20^{+}$. Three months of treatment with 50mg of vitamin B6 improved vitamin B6 and $CD4^{+}$ lymphocytes. The concentration of IgG, IgM, and IgA was not found to have any association with vitamin B6 treatment. This study reported that vitamin B6 deficiency is common in individuals with CVID. However, its supplementation did not improve humoral immunodeficiency[49,62-63].

Vitamin A

Vitamin A deficiency is a predisposing factor for infections of the GI and respiratory tracts[64-65]. In patients with immunodeficiency, deficiency of this vitamin has an immunomodulating effect. CVID individuals were found to have low vitamin A levels compared to healthy individuals. CVID individuals with malnutrition, bacterial infection, and splenomegaly individuals have lower vitamin A levels [66-67]. However, whether this deficiency can modulate the onset of disease or modulate its progression and/or associated pathologies is unclear. The administration of vitamin A (6500 IU per day), for six months, increased vitamin A levels in CVID individuals. Furthermore, it was associated with an increase in the concentration of the anti-inflammatory cytokine, IL-10, and a decrease in the pro-inflammatory cytokine, TNF-α. The supplementation of vitamin A enhanced IgA levels. However, no significant changes in IgM levels were observed. This study supported

the contention that vitamin A supplementation enhances anti-inflammatory cytokines and immunoglobulin levels while suppressing pro-inflammatory cytokines, in CVID individuals[66].

Vitamin B12

B12 deficiency is considered a cause of impaired immunoglobulin production[68], and both vitamin B12 and folic acid imbalance modify NK cytotoxicity, B lymphocytes and lymphoproliferation in mouse models[69].

Vitamin D

Paulino *et al.* examined vitamin D levels and found a high percentage of 25-hydroxyvitamin D deficiency in the Ataxia-telangiectasia group (6/14; 42.8%) and CVID group (3/17; 17.6%)[70].

Vitamin E

In two case series, it was speculated that patients with CVID and enteropathy might be relatively susceptible to developing symptomatic vitamin E deficiency. This report suggested that vitamin E deficiency may be a potential factor of unexplained neurological disease with retinitis pigmentosa in 5 out of 14 patients in PAD[51]. In addition to these case series, it has been reported that lower serum level of vitamins A and E are present in patients with PID, but not for vitamin D. Also, in PIDD, the decreased serum vitamin A levels were positively correlated with reduced IgG levels. However, there was no significant association between their immunological specifications and vitamin E levels[71].

Zinc

Acrodermatitis enteropathica and SCID-like syndrome are features associated with zinc deficiency[64]. Comparison of different levels of vitamin A and zinc found that CVID patients had lower levels of retinol and zinc than controls. This deficiency may contribute to their disease severity leading to a state of chronic inflammation. Therefore, it may be recommended that micronutrient status is always checked in patients with primary immunodeficiency[64].

1.3.3. Microbiome as an immunomodulator

Few studies have shown that the microbiota may activate and help to condition host immunity. The relationship between altered gut microbiota and the PIDD, especially CVIDs, was more explicit and linked to autoimmunity conditions in a few studies[54,72]. The expression of different B-cell

phenotypes was studied together with gut microbiota in mice models. Data showed that the gut microbiota boosts regulatory B cells differentiation in both the spleen, and the mesenteric lymph nodes[73]. Figure 5 explains some of the association between the microbiome and CVID.

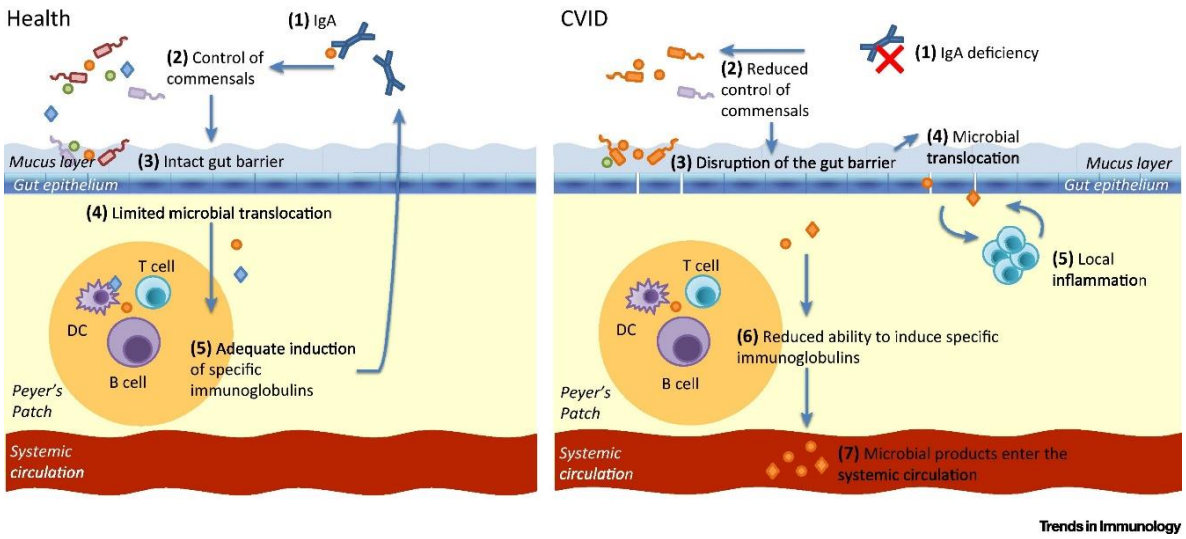


Figure 5a: Control of the microbiome in health (left panel) and in CVID (right panel).

The mechanisms leading to immune dysregulation in a normal control of the microbiome (left panel) and the reduced control of the microbiome in CVID (left) (Abbreviations: CVID, Common variable immunodeficiency; DC, dendritic cell).

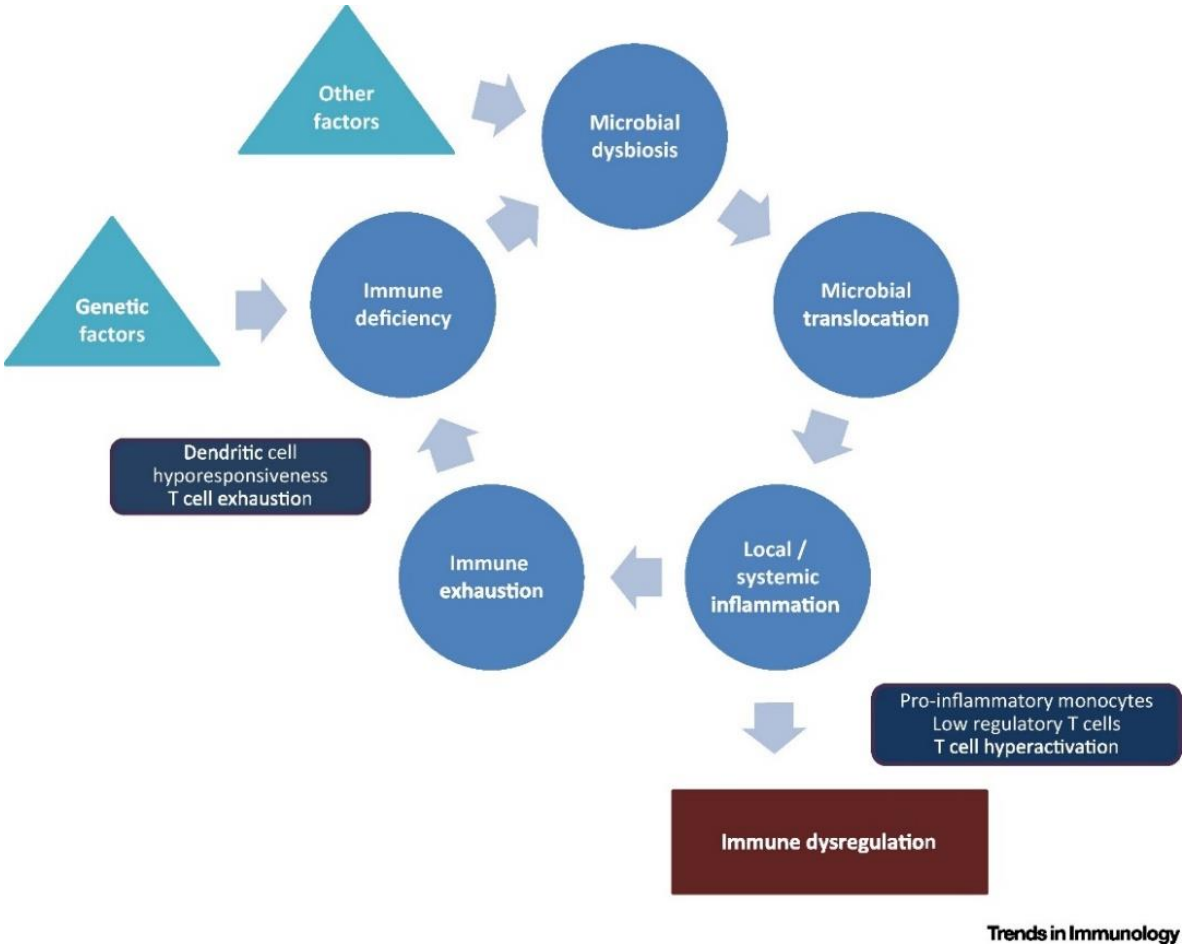


Figure 5b: Factors leading to immune dysregulation in CVID.

The reduced control of the microbiome in CVID is one of the factors that may lead to immune dysregulation.

Figure 5.0: Microbiome in health and disease (CVID) and mechanisms leading to immune dysregulation.

Susceptibility to CVID in combination with other factors, may lead to microbial dysbiosis and can lead to a vicious cycle leading to inflammation and a state of immune dysregulation (*Note: adapted from Berbers et al. 2017* [54]).

1.3.4. miRNA may potentially be a modulator and a target for preventative and therapeutic options

miRNAs are small molecules (21-25 oligonucleotides) that regulate gene expression, at the stage following transcription, and may lead to mRNA degradation and the inhibition of translation[74-75]. They are secreted in exosomes, other microvesicles, and lipoproteins from different cells types[76-77] into the circulation and may also act as biomarkers of diseases[78]. The use of miRNAs is under investigation as part of an attempt to produce personalised therapies like miRNA antagonists[79] and transferring specific molecular functions to cells that receive exosomes[80] (Table 3). There have been a few reports in animal models of miRNAs playing a role in the immune system[81].

1.3.5. The development of *"Triad Interaction Theory"* (miRNA, microbiome, and micronutrients) for PIDD patients

There have been no previous studies of dietary change, causing disease modulation in patients with PIDD. Also, no studies have been reported evaluating the relationship between miRNA and gut microbiome in patients with PIDD.

Targeted microRNA	Disease	Mechanism	Company	Clinical Stage
miR-122	Hepatitis C virus infection	Anti-miR: block HCV infection	Regulus Therapeutic	Preclinical
		Anti-miR: Prolonged mean reductions in viral plasma RNA levels from baseline	Santaris Pharma Mirna Therapeutics	Phase IIa
miR-10b	Glioblastoma	Anti-miR: ↓ proliferation by blocking cell cycle progression and triggering cell death	Regulus Therapeutic	Preclinical
miR-221	Hepatocellular Carcinoma	Anti-miR: delayed tumour progression resulting in enhancing the survival rate.	Regulus Therapeutic	Preclinical
miR-21	Renal fibrosis	Anti-miR: ↓ the expression of extracellular proteins.	Regulus Therapeutic	Preclinical
miR-33	Atherosclerosis	Anti-miR: regulation of cholesterol and fatty acid homeostasis (↓very low-density lipoprotein triglyceride & ↑ high-density lipoprotein	Regulus Therapeutic	Completed Preclinical
miR-34	Solid liver Cancer (primary or secondary)	Mimic: ↓ the expression of oncogenes, enhanced the survival, ↓growth of non-hepatic tumour.	Santaris Pharma Mirna Therapeutics	Phase I
miR-155	Haematological malignancy	Anti-miR: restores normal function and ↓ aberrant cells proliferation	Santaris Pharma Mirna Therapeutics	Completed Preclinical
miR-92	Peripheral artery disease	Anti-miR: enhance blood vessel growth and improve functional recovery of damaged tissue.	miRagen Therapeutic	Preclinical
miR-15	Myocardial infarction	Anti-miR: ↓ death and promote regeneration of heart muscle cells.	miRagen Therapeutic	Preclinical

Table 3: MicroRNA therapeutic development.

Several miRNAs are currently at the preclinical and clinical stages and may soon be available for use in humans. (*Note: adapted from Christopher AF et al., 2016)*

1.4. Summary

Primary Immunodeficiency, particularly predominantly antibody deficiency, is a group of disorders that predispose affected individuals to body organs' destruction and complications (bronchiectasis, autoimmunity, malignancy, and allergy) and result in lifelong disabilities and a decreased quality of life. The relationship and association between immune cells, miRNA, microbiome, and micronutrients are not well established in the literature. Studying this relationship was the aim of this project. Identifying novel biomarkers and meaningful stratification may lead to a better understanding of the aetiology of the disease, thereby helping to personalise effective therapy and improve monitoring methods. The detailed results (miRNA and microbiome) from this study may lead to better stratification and care.

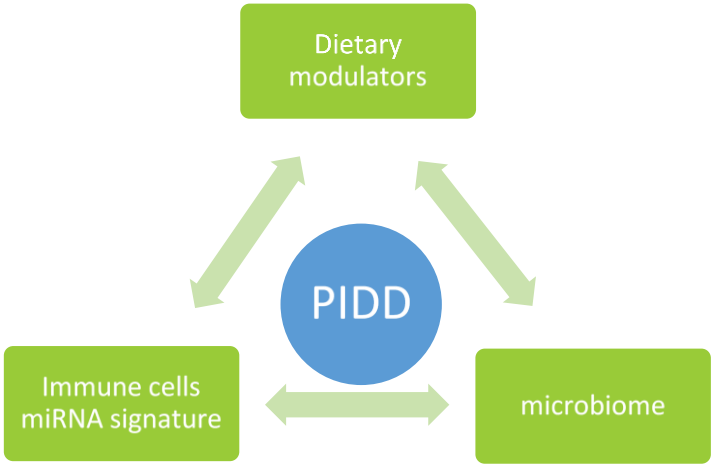


Figure 6: The interaction between immune cells, microbiome and dietary modulators in patients with primary immunodeficiency disorders.

1.5. Aims and objectives of the project

1.5.1. Research hypothesis

Current literature shows that there is a relationship between diet, miRNA signature in immune cells, and the microbiome. Patients with PIDD (either causally, or as a consequence) appear to have different microbiome profiles associated with the disease. Equally, changes in miRNA may be related to these changes, and the symptoms and prognosis of patients with PIDD may be influenced through and by dietary modification. Therefore, the study proposed to investigate the following aims and objectives.

1.5.2. Aims and Objectives

1.5.2.1. <u>The main objectives were to investigate the following</u>

- The interaction between immune cell phenotype and the gut microbiome in patients with B-cell diseases prior to any intervention
- The effect of, either alone or combined, the commencement of intravenous immunoglobulin (IGIV) (short- and long-term effects) and supplementation with micronutrients (multivitamins) on:
  - Patients' symptoms, by measuring clinical, physiological and biochemical markers and by conducting symptoms and dietary questionnaires before, during intake and after stopping MVT to evaluate other dietary effects and the improvement of patient symptoms and safety.
  - Immunological cell signatures, exosomal micro RNA, and gut microbiomes.

1.5.2.2. <u>Secondary objectives were to investigate</u>

- Correlations between circulatory cytokines, vitamins levels, and the microbiome in patients with PIDDs with the clinical diagnostic disease and different phenotypes.
- Regrouping (re-stratifying) B-cell PIDDs based on phenotypes, diagnosis and lymphocytes' signature.
- Relationships between the triad (micronutrients, microbiome, and immunodeficiency) and patient presentations and symptoms.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Study plan and design

The following scheme **(Figure 7)** represents the steps that will be carried out in this research study.

Study Design

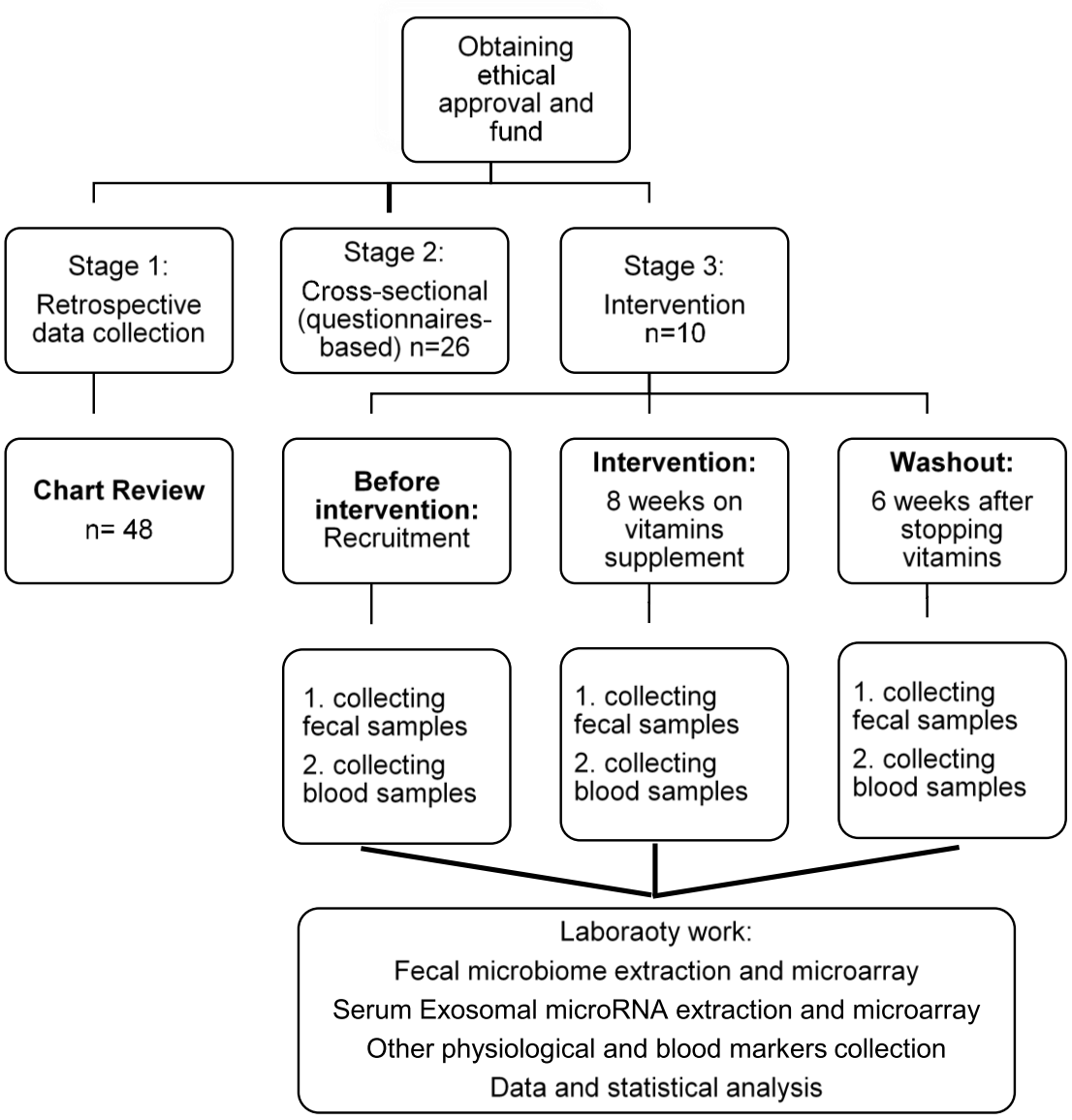


Figure 7: Summary of the study design.

The study is composed of three parts. The first stage is a chart review of patients with PIDD who were followed in immunology clinics. The second stage is conducting a questionnaire-based cross-sectional study, and the third stage is selecting ten patients for the intervention.

2.1.2. Ethical approval and funding application

The process of obtaining ethical approval for the research started directly following the receipt of the offer for the MRes by the University of Bristol in September 2017. It is consistent with ensuring that the human rights of research participants and researchers were both protected. The guidelines and policies provided by the Department of Medicine, Pulmonary and Allergy Division Medical Research Centre (MRC) in Hamad Medical Corporation (HMC) were followed during the application process, and official documentation was produced. These guidelines are in alignment with the University of Bristol Research guides. A full proposal (13 pages) was submitted, with two consent forms (Informed consent and Assent consent- English and Arabic forms), data collection sheet and the two questionnaires that were to be used in the study. This process took three months of preparation and submission. The papers passed the MRC scientific review and were approved in November 2017.

Following the approval of the MRC, papers were transferred to the Institutional Review Board (IRB) (Research Ethics Committee) of the HMC to deal with the application in accordance with the agreed local policy, procedures and regulations governing human subject studies. Six weeks were required for final approval. The application received final approval in December 2018. The study was awarded a grant of 19,592.76 GBP in January 2018. An amendment to exceptional upgrade of the budget to reach around 30,300 GBP was made and received approval in April 2018. The final release of the amended approval was received, and the purchases for the materials and consumables were requested. See appendices I and II for further details of the grant (budget), and ethical approvals.

The plan of investigation is shown in Figure 7.

2.1.3. Data Collection

Data were retrospectively collected from medical records for all patients attending and followed up in the adult Immunology Clinic, Hamad Medical Corporation Qatar, over the last ten years. All patient records that fulfil the criteria for primary immunodeficiency were reviewed. The patients' B-cell phenotypes and complications associated with each disease were further characterised.

2.1.4. Patients Recruitment

A cohort of 10 patients affected by PIDD (Predominantly (B-cell) antibody or combined immunodeficiency), with a focus on CVID and SAD, attending the Unit of Adult Allergy and Clinical Immunology at Hamad Medical Corporation, Qatar were included. PIDD (CVID and SAD) diagnosis was based, with a minor modification, on the ESID 2017 criteria. The post-vaccination poor response was defined as anti-pneumococcal polysaccharide antibody level that did not reach a level of 0.35 µg/mL after vaccination in 50% or more of the serotypes. The following criteria were used to diagnose CVID: low IgG levels (less than 2 SD below the mean for patients' age), and low IgM or IgA. In addition, the involvement of any of the following was used: an onset of immunodeficiency after two years of age, poor response to vaccines or the exclusion of other defined causes of hypogammaglobulinemia[82-83]. For SAD, criteria for diagnosis include a combination of the following: Infections (recurrent or severe bacterial, normal serum/plasma IgG, A and M and IgG subclass levels, profound alteration of the antibody responses to *S. pneumoniae* (or other polysaccharide vaccine) either after documented invasive infection or after test immunisation and the exclusion of T cell defects[83]. Inclusion criteria were immunologist-diagnosed PIDD (Predominantly (B-cell) antibody or combined immunodeficiency), age range 12-65 years, patients with PIDD who have been followed up for at least the last three months, and non-smokers over the previous eight weeks before the intervention. Exclusion criteria included pregnant women and subjects suffering currently from certain terminal illnesses, for example, cancer (and on chemotherapy), and subjects who refused or were unable to give informed consent, for example due to the presence of other neuropsychiatric conditions, namely autism and mental impairment. Then, selected patients were contacted by telephone, and an appointment was made. Patients were evaluated clinically during the clinical visit (Figure 8). As this was a proof of concept study, a sample size of ten patients was considered sufficient.

Flow chart for the recruitment strategy

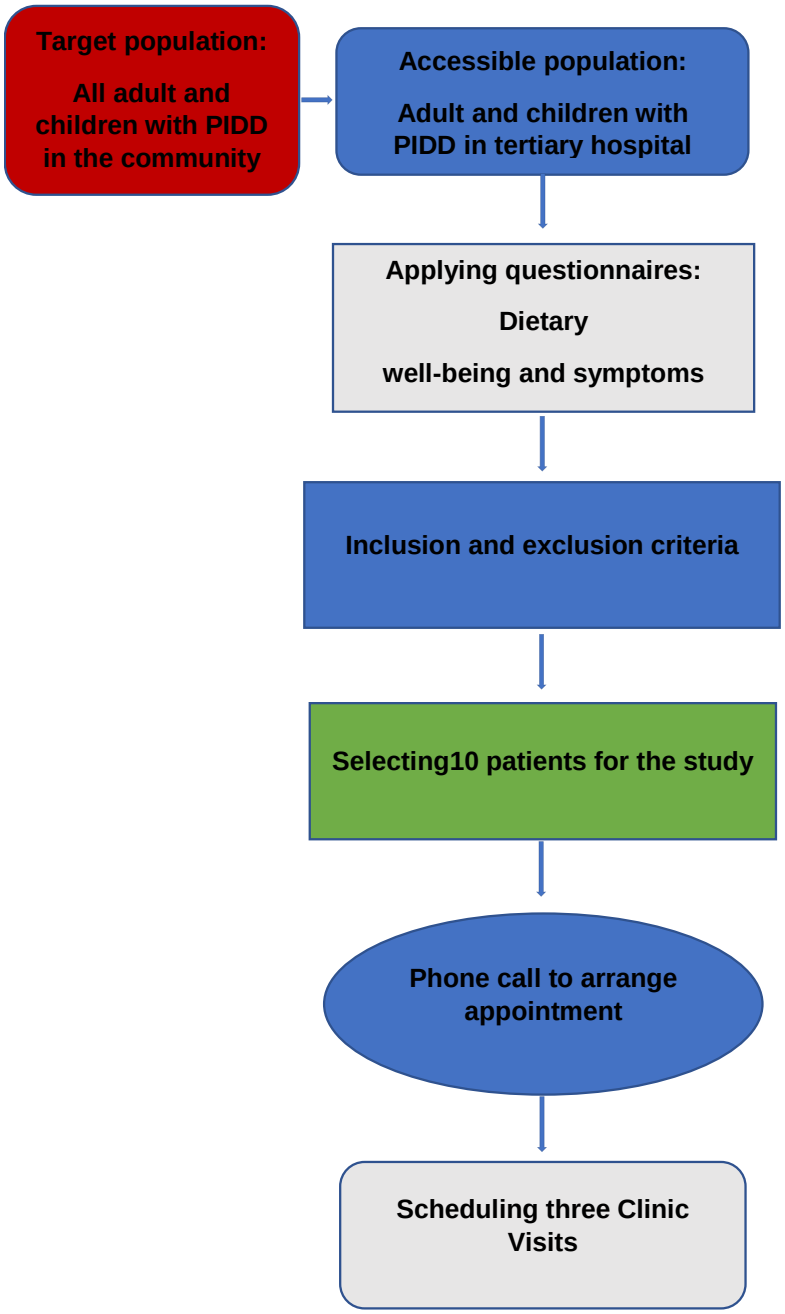


Figure 8: Flow chart of the patients' selection and recruitment.

For stage 3 (interventional study), the scheme of steps that were followed is shown.

2.1.5. Designing the Questionnaires

Dietary habits, health, and wellbeing questionnaires before recruitment were utilised to help to perform a subjective assessment of the study patients. A Symptoms Questionnaire was used to evaluate the patients' symptoms and wellbeing before recruitment and as a measuring tool for reassessment during the study period. A Dietary Questionnaire was used to evaluate patients' dietary habits before starting the study and to monitor any major changes in dietary habits that may affect the intervention step. The dietary questionnaire that was developed was based on the Harvard Food Frequency Questionnaire (for food habits). The wellbeing and symptom questionnaires that were developed was based on health quality SF6 and CH50 (for health and clinical stability)[84-87].

These Questionnaires were in English and translated into Arabic with validation of the translation by two expert physicians in the field of allergy and immunology, with an internationally accepted competency in English. Retranslation into English was done to confirm the accuracy of the translation. Validation of the questionnaires was done using a standardized procedure which included pilot-testing in the allergy and immunology unit for different types of patients, followed by psychometric validation by asking the participants to answer the questionnaire twice, to assess test-retest reliability. Also, the symptom questionnaire was applied for selected patients during the development of sickness (Figure 9). It was estimated that 40 minutes was needed to conduct the diet and symptoms questionnaires. (Appendices III-IV).

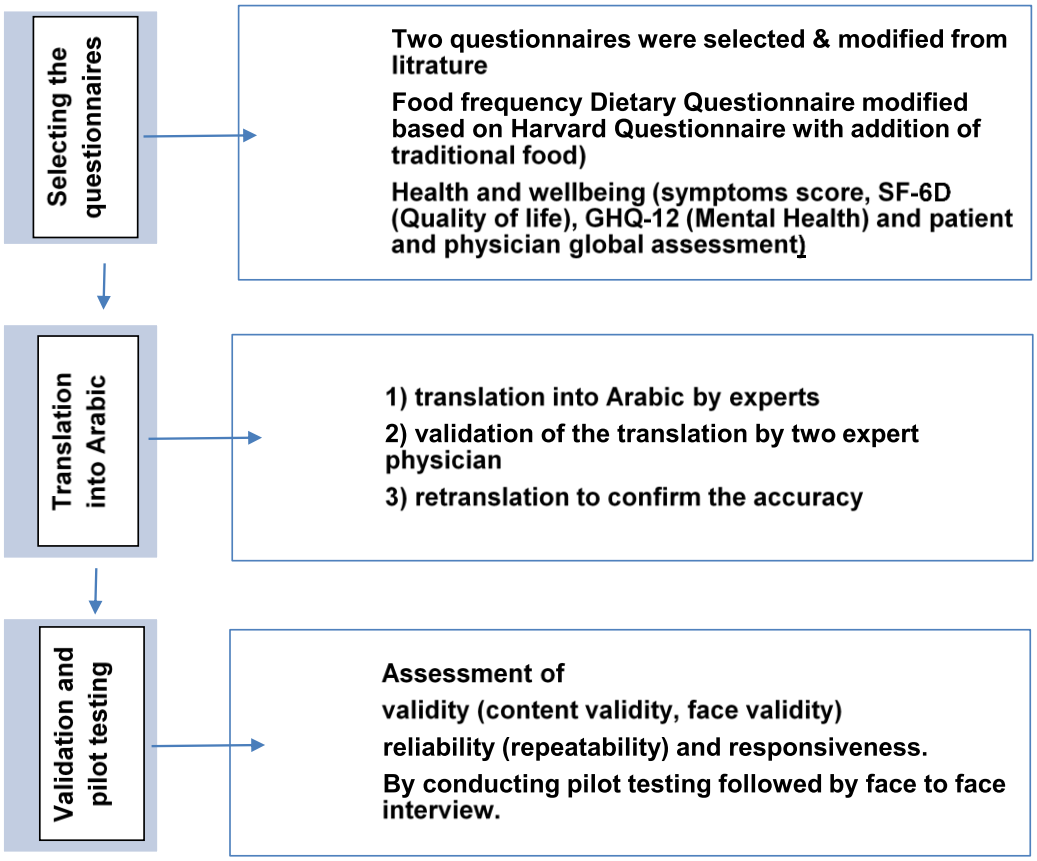


Figure 9: Development and validation of the questionnaires.

2.1.6. Participants and Samples Collections

2.1.6.1. Blood and stool samples

Patients with the diagnosis of PIDDs and fulfilling the inclusion criteria were investigated: Ten patients were selected for the diagnosis of humoral PIDD. Blood and stool sample collections were performed on four occasions over the study period: 1st sample before introducing IGIV, 2nd sample before vitamins interventional step, 3rd sample after the eight weeks of intervention, and 4th sample 6-8 weeks after stopping the intervention. Other blood samples were collected to evaluate vitamin levels as part of the usual care and repeated once during the interventional step and if patients developed any new symptom that suggested vitamin toxicity. Vitamin medication was considered a safe treatment. In case the blood test was suggestive of the presence of severe symptomatic vitamin deficiency, patients were provided with a proper management plan, including referral to an appropriate speciality to check for secondary causes and provide treatment. Other anthropometric measurements like weight (kg), height (m), BMI (kg/m^2), BP (mmHg), and physiological markers including spirometry (FEV1, FVC, and FEV1/FVC) and FeNO were collected during the three visits. CBC, CMP, and lipids profiles were checked during the four visits (Figure 7).

2.1.6.2. Interventional Step

The patients were given an internationally accepted and recommended dose of multivitamin supplements for an eight-week duration (intervention period). Vitamin supplementation type: A, E, B & D tablets. Doses were: Vitamin A 30,000 IU & E 70 mg (combined tablet) once daily, vitamin B complex (B-1 thiamine 5 mg, B3 Nicotinamide (NAM) 20mg, B-2 riboflavin 2 mg, and B-6 pyridoxine 2 mg) once daily & Vitamin D2 50,000 IU once weekly. Blood and stool samples were collected at each stage (Figure 7).

2.2. Methods

2.2.1. Retrospective Chart Review

Clinical Data of patients with PIDD registered over the last 10 years, in the adult allergy and immunology clinic, Hamad General Hospital, HMC, will be reviewed from Cerner electronic and physical medical records. Ethical approval and fund were granted from HMC-MRC as described in section 2.1.2.

2.2.2. Laboratory work

2.2.2.1. Samples collection and processing

Samples were collected in 3 different tubes (whole blood EDTA, Serum yellow-toped, and PAX gene tubes (Qiagen, Plymouth, UK)). A comparison was done between total and small RNA species extracted from serum exosomal, PAX tubes, and blood lymphocytes buffy coat with multiple trials to assess the best blood samples to be utilised for the study. Three extraction kits were used to compare the best quality of small RNA species extraction from serum exosome. (Appendix V)

For microbiome DNA, two extraction kits were compared to identify the best kit producing good quality DNA for future extractions. Stool samples were collected in regular collection tubes and then transferred immediately upon receipt from patients to special OMNigene tubes (details below). Stool samples were stored at room temperature for a maximum of one week before extraction (may be kept at room temperature for up to 60 days). See Appendix (V) for the supplementary supportive initial experimental data.

Whole blood samples were collected in yellow top vacutainer tubes (BD, SST™ Tubes contain spray-coated silica and a polymer gel for serum separation). Blood samples were left at room temperature for one hour to allow complete coagulation. Coagulated samples were then centrifuged at 2,000 rpm using SL 16R Centrifuge (Thermo Fisher Scientific Robert-Bosch-Straße 1 D - 63505 Langenselbold Germany), for 10 minutes at room temperature to separate the serum. Serum was transferred to a new RNA free microcentrifuge tube with care taken to not disturb the buffy coat. Serum samples were immediately frozen at −80°C until exosome RNA extraction.

2.2.2.2 Serum Exosome microRNA Extraction

Different miRNA extraction methods from serum exosomes were evaluated with control serum samples. The extraction methods, mirVANA miRNA Isolation Kit (Ambion, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), TRIzol Reagent (Invitrogen, Life Technologies, Grand Island, NY, USA), and TRIzol LS Reagent (Invitrogen), were tested with or without a carrier, ie glycogen (Invitrogen). Extractions were also performed using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA; see Appendix V).

Extraction of exosome microRNA from serum was done using the Invitrogen extraction kit (Invitrogen, Life Technology, Carlsbad, CA, USA). Here, 500 µL of serum was used to precipitate

a pellet that was then re-suspended in 500 µL PBS. miRNA was extracted from exosomes suspension solutions by miRNeasy Mini Kit (Qiagen, Valencia, CA, USA), where 200 µL of the sample was used per extraction.

2.2.2.3. Microbiome DNA Extraction from stool

For stool samples, extraction of DNA was done using two extraction kits (QIAamp PowerFecal DNA Kit (Cat No./ID: 12830-50 and QIAamp Fast DNA Stool Mini Kit (50): 51604 Qiagen Germany). Special collection tubes of stool were used (OMNIgene.GUT faecal collection kits; OM-200) to help with preserving the faecal samples for longer before extraction. As the extraction using QIAamp PowerFecal DNA Kit was of better quality, subsequent samples were extracted by this method only for running the array plates. A clean-up method using multiple ethanol washes and a heating step at 37ºC for a few minutes were added to improve the quality of the extracted DNA, as per the manufacturer's requirements. See below for microbiome DNA quality assessment.

2.2.2.4. Assessing the quality of serum exosomal miRNA and stool microbiome DNA

2.2.2.4.1. miRNA Quality

miRNA quantity and purity were assessed by spectrometric analysis using the Nano-drop (ThermoFisher Scientific NanoDrop™ 2000/2000c Spectrophotometers). RNA concentration and integrity were evaluated by the gel electrophoresis using the Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany). The samples that passed the above quality assessment tests were selected for further analyses. RNA with RIN > 6 were selected, while samples with RIN <3 were considered degraded, with miRNA sizes in the region 10-40 nt. The average size was used in pg/µl, with good miRNA /small RNA fraction. The RNA ladder was 50-300bp (Figure 10a).

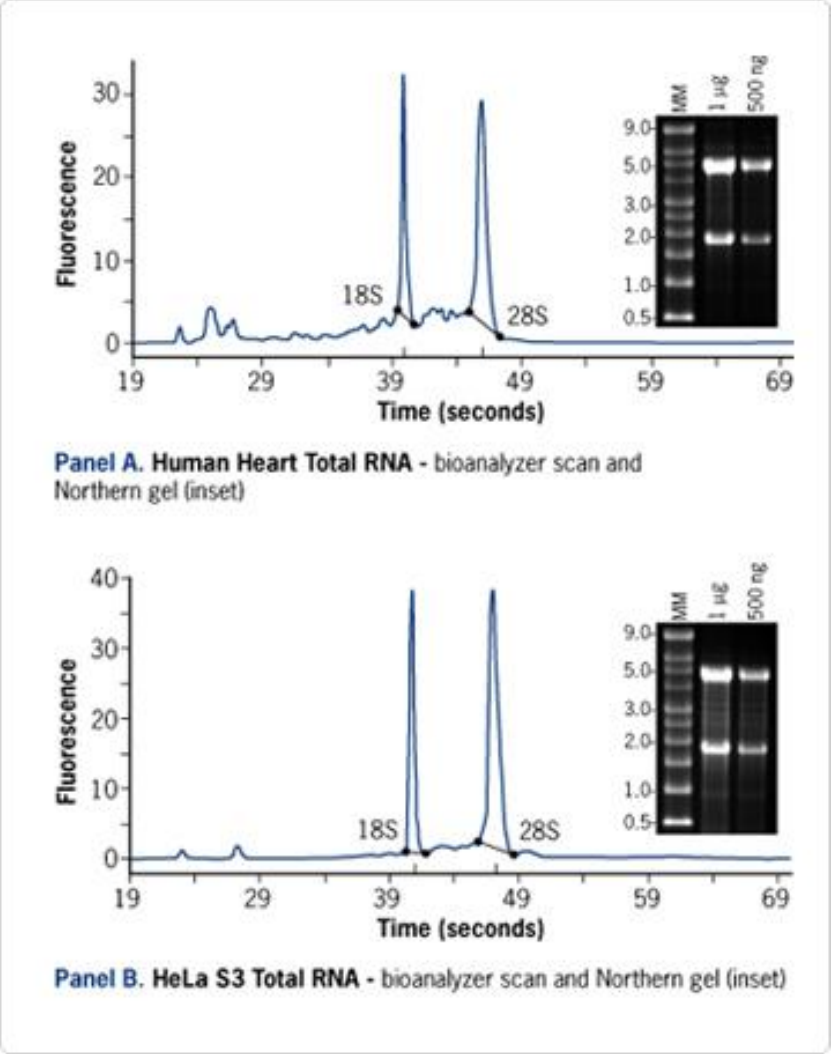


Figure 10a: RNA integrity and concentration using Agilent bioanalysers.

Adapted from ThermoFisher Scientific website; accessed 13/04/2018. (https://www.thermofisher.com/qa/en/home/references/ambion-tech-support/rna-isolation/tech-notes/assessing-rna-quality.html)

Small RNA (200 nt) were analysed by using kits from Agilent Technologies and chip technology. Fragments with a size of 15–40 nt were defined as miRNA (See figure below). The concentration of miRNA was calculated as absolute amount [pg] and as a percentage of small RNA [%]. (Figure 10b).

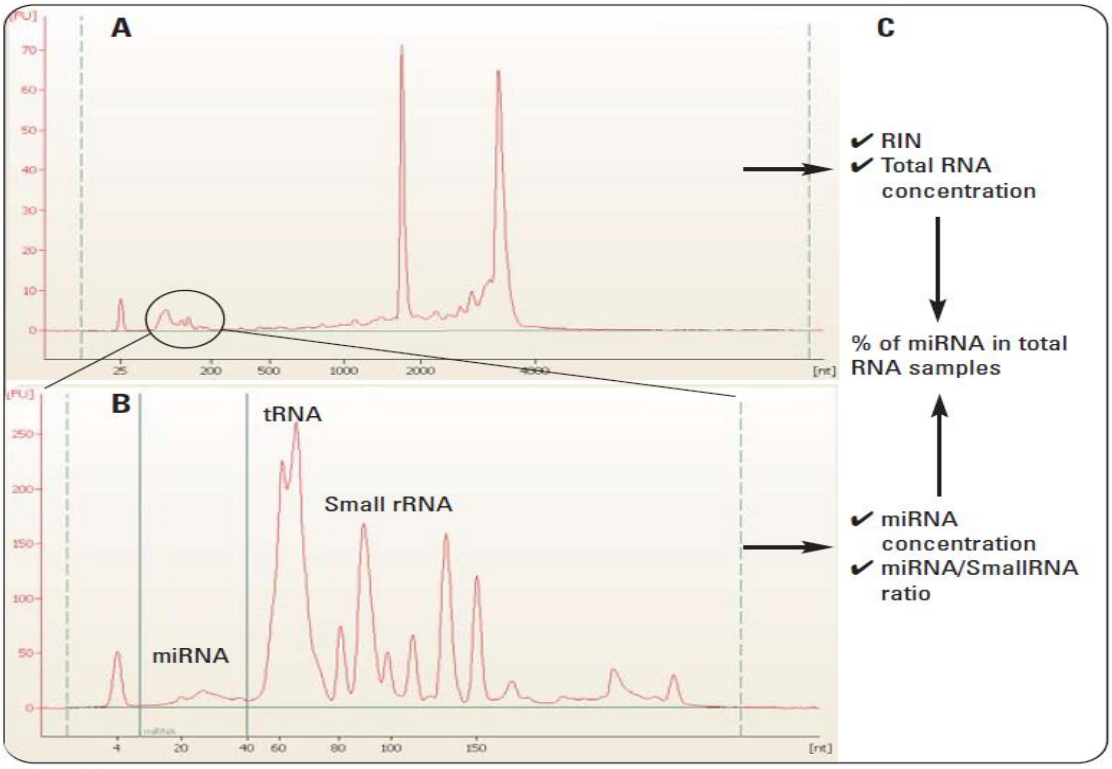


Figure 1
Total and small RNA analysis. A) The electropeherogram shows the total RNA pattern analyzed with the RNA 6000 Nano assay. B) The electropherogram shows the small RNA pattern analyzed with the Small RNA assay. C) miRNA analysis workflow. All RNA quantities were used to estimate the % of miRNA in total RNA samples.

Figure 10b: Electropherogram for small RNA analysis.

Example of electropherogram for small RNA analysis performed with the small RNA assay on the 2100 bioanalyser (Agilent Technologies).

Adapted from ThermoFisher Scientific website; accessed 13/04/2018.

(https://www.agilent.com/cs/library/applications/5989-7870EN.pdf)

2.2.2.4.2. Microbiome DNA Quality Assessment

2.2.2.4.2.1. Genomic DNA for microarray

Prior to the axiom assay, the quality of the genomic DNA was determined to be double-stranded, of high purity, free of DNA polymerase inhibitors (e.g. haem and EDTA), and salt-free to avoid inhibiting enzyme reactions. DNA purity was assessed by OD260/OD280 and OD260/OD230 ratios. The OD260/OD280 ratio should be between 1.8 and 2.0 and the OD260/OD230 ratio should be greater than 1.5. A clean-up step was done to reach these required ratios. Microbiome quantity and purity was assessed by spectrometric analysis using the Nanodrop (ThermoFisher Scientific NanoDrop™ 2000/2000c Spectrophotometers)

To ensure that the DNA was not degraded, the average size of gDNA was assessed by electrophoresis using 1% agarose gel and appropriate size standard controls. Approximately 90% of the DNA must be greater than 10 Kb in size. See Figure 10c for examples of intact and degraded gDNA.

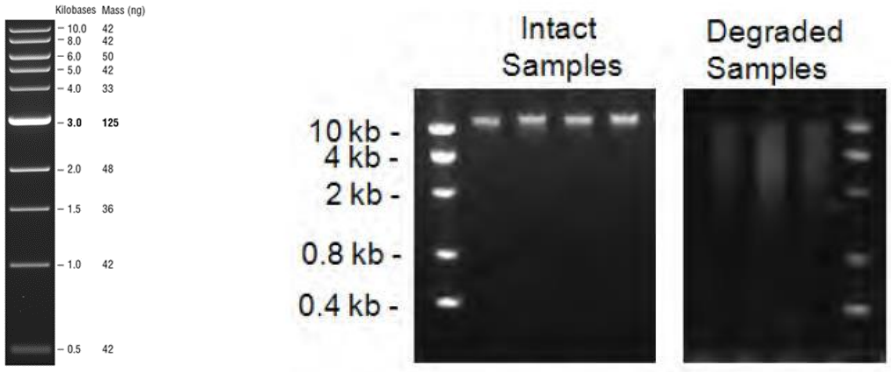


Figure 10c: Agarose E-gel images of intact genomic DNA.

Figure shows example of 1% agarose E-gel images of intact genomic DNA, suitable for use in the Axiom 2.0 Assay. Degraded gDNA was deemed not suitable for use. Approximately 90% of the DNA must be greater than 10 Kb in size.

Adapted from ThermoFisher Scientific website; accessed 13/04/2018. (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/703335_Axiom_24F_ManualWrkflw_UG.pdf)

2.2.2.4.2.2. Fragmentation QC checks using 4% agarose E-gel

During microarray preparation, Stage One of DNA amplification was followed by Stage Two of fragmentation, precipitation, and Stage Three of centrifuge and drying, resuspension and hybridisation preparation. Before proceeding to Stage Four of Denaturation and hybridisation, it was highly recommended to perform quantitation and fragmentation quality control checks at this stage using 3-4% agarose E-gel. See below figure 10d for an example of QC checks.

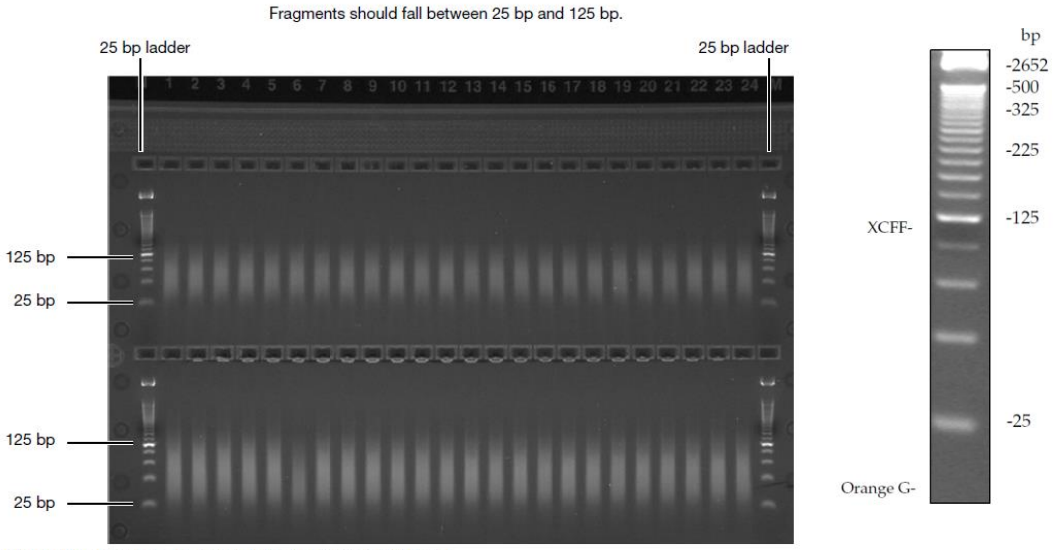


Figure 50 Example of a typical fragmentation QC E-gel

Figure 10d: Agarose E-gel of fragmentation QC checks.

Example of 4% agarose E-gel of fragmentation QC checks with the ladder used. Fragments should fall between 25bp and 125bp.

Adapted from ThermoFisher Scientific website; accessed 13/04/2018. (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/703335_Axiom_24F_ManualWrkflw_UG.pdf)

Figure 10.0: Quality assessment of small RNA and genomic DNA.

Figure 10a: RNA integrity and concentration using Agilent bioanalysers.

Figure 10b: Electropherogram for small RNA analysis.

Figure 10c: Agarose E-gel images of intact genomic DNA.

Figure 10d: Agarose E-gel of fragmentation QC checks.

2.2.2.5. Samples selection for microarrays

Only samples that passed the good quality assessment were selected for running on the array plates.

2.2.2.6. Microarrays

2.2.2.6.1. Serum microRNA expression profiling using Array Plate

The plate used in the microarray was Affymetrix® miRNA 4.1 24 Array Plate. The manufacturer's instructions were followed in all protocols except for oven incubation, which was increased to 42 hours, instead of 17 hours.

2.2.2.6.1.1 Label Biotin: Affymetrix® FlashTagTM Biotin HSR RNA Labelling Kit to label the samples were used. A brief tailing reaction was followed by ligation of the biotinylated signal molecule to the target RNA sample (Figure 11).

2.2.2.6.1.2 Hybridization protocol: Hybridisation was conducted according to the manufacturer's instructions as per the GeneChip miRNA 4.1 Human Array Plate/FlashTag™ Biotin HSR RNA kit (Affymetrix, ThermoFisher Scientific).

2.2.2.6.1.3 Scan protocol: Arrays were scanned with GeneAtlas Imaging station scanner (Affymetrix, ThermoFisher Scientific).

2.2.2.6.1.4 Data processing: The data was processed using Affymetrix expression console and evaluated by Affymetrix transcription console software (Affymetrix, ThermoFisher Scientific). RMA algorithm.

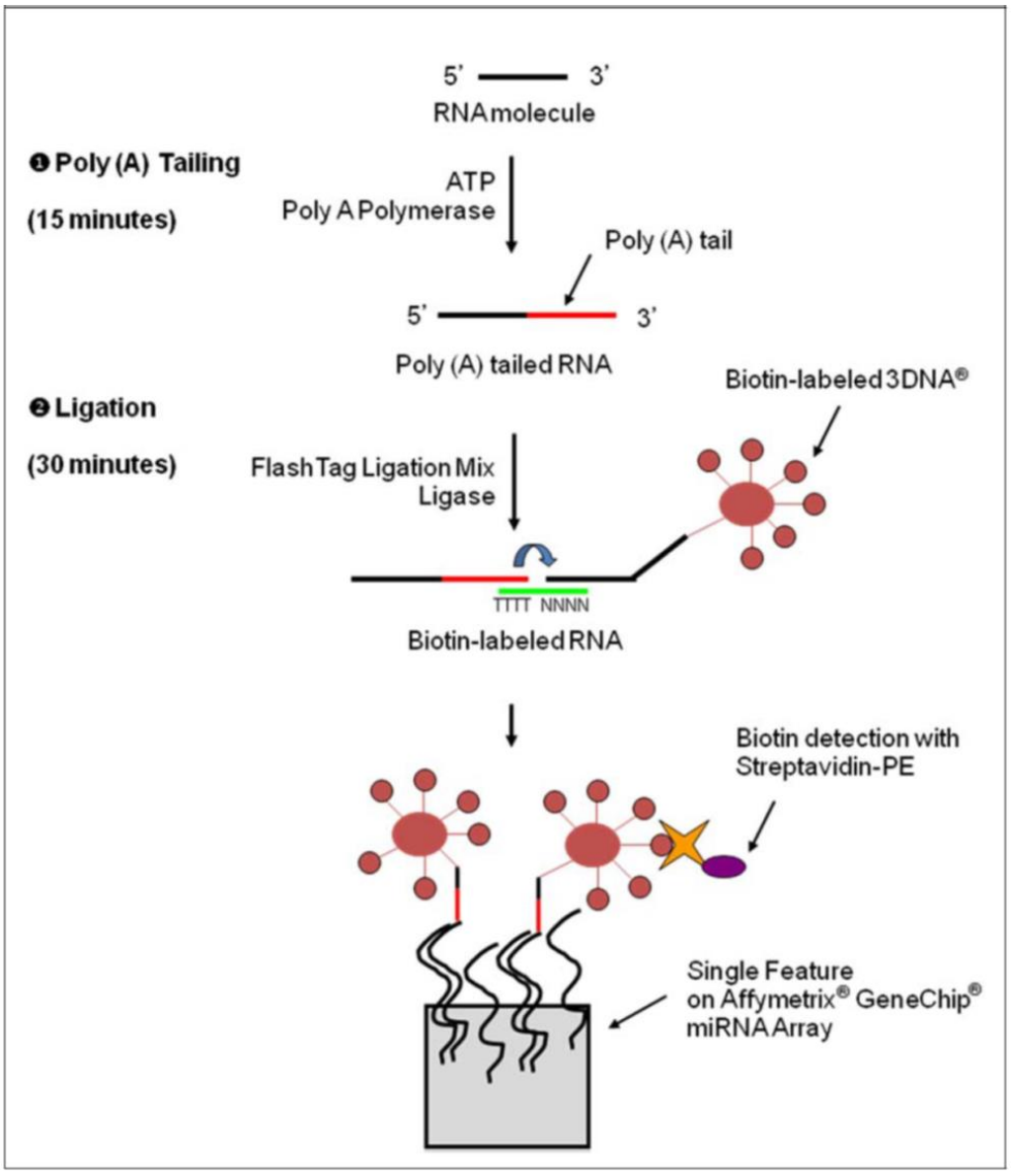


Figure 11: FlashTag™ HSR: Procedure overview.

For analysis by Affymetrix GeneChip miRNA Arrays, it is essential to use a specific protocol for labelling total RNA or low molecular weight (LMW) RNA using FlashTag™ Biotin HSR Labelling Kit that includes a brief tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA sample. The test takes less than one hour.

Adapted from ThermoFisher Scientific website; accessed 16/03/2019.

(https://media.affymetrix.com/support/downloads/manuals/mirna_flashtag_manual.pdf)

2.2.2.6.2. Microbiome Arrays

Different methods exist for microbiome sequencing. Three microbiome sequencing methods were compared in a recently published study by Thissen JB *et al*. (2019) [88] (Table 4).

Features	16S rRNA sequencing	Metagenomic sequencing	Axiom microbiome
Detect sequenced bacteria	Y	Y	Y
Detect sequenced viruses	N	Y	Y
Detect sequenced fungal, archaea, protozoa	N	Y	Y
Detect un-sequenced microorganisms	Y (bacteria only)	Y	N
Detect Functional genes	N	Y	N
Species resolution	N	Y	N
Strain resolution	N	Y	N
What is detected	16S rRNA gene	Genome fragments	Genome fragments
Length of DNA detected (DNA bases)	~400	~150-300	~35
Instruments	Illumina MiSeq, Thermo Fisher Ion Torrent	Illumina NextSeq, NovaSeq, HiSeq	Thermo Fisher GeneTitan
Instrument cost	++	++++	+++
Time on instrument (hours)	24-36	26-264	48
Per-Sample reagent cost	$30-50	$300-500	$40-50

Table 4: Sequencing methods utilised in the literature to identify the human microbiome. Comparison between three methods: Axiom Microbiome Array, 16S rRNA sequencing, and the metagenomic sequencing. The Axiom Microbiome can detect as small as 35 DNA bases. It is unable to detect un-sequenced microorganisms when compared to the 16S rRNA sequencing and the metagenomic sequencing[88]. However, the Axiom Microbiome array is based on the recent sequence data and more probes per target compared to other microbial detection arrays, and it covers more bacterial and viral targets (*Note: adapted from Thissen et al. 2019 [88] and Gardner et al. 2010 [89]*).

Dilution of DNA samples was done to 5 ng/µL for human DNA samples in order to run the arrays according to the manufacturer's requirements. The DNA extracted from faecal samples was applied to a microarray designed to detect all known microorganisms in a sample, with family-, species- and strain-level identification**.** Axiom™ Microbiome, 24 array plates, Catalogue number: 902903, ThermoFisher Scientific was used for this purpose. See appendix VI (A-H) for procedure details. The table below summarizes the target categories represented in the Axiom MiDAS database.

Domain	Number of families	Number of species	Number of target Sequences
Archaea	31	370	606
Bacteria	278	6,901	34,254
Fungi	121	381	658
Protozoa 30 91 229	Protozoa 30 91 229	Protozoa 30 91 229	Protozoa 30 91 229
Virus	100	4,770	99,808
Total	560	12,513	135,555

Table 5: Axiom™ MiDAS target sequence database.

This table of Axiom MiDAS target sequence database is comprised of select entries exported from Lawrence Livermore National Laboratory's KPATH pathogen surveillance pipeline and database (https://str.llnl.gov/str/April04/Slezak.html). The KPATH pipeline automatically and regularly checks public domain sequence repositories (e.g., NCBI) for new and relevant sequence entries and incorporates them into the database. Axiom MiDAS database contains KPATH records that were current as of October 2014. (Adapted from Axiom™ Microbiome Solution USER GUIDE Catalogue Numbers 902910, 902904, and 902903- Publication Number 703408.

Adapted from ThermoFisher Scientific website; accessed 16/03/2019 (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2F703408_Axiom-Microbiome-UG.pdf&title=VXNlciBHdWlkZTogQXhpb20gTWljcm9iaW9tZSBTb2x1dGlvbg==)

2.2.2.7. microRNA and microbiome Genetitan, Axiom MiDAS and transcriptome analysis console course training.

(See appendix (VII) for training certificates)

2.3. Statistical Analysis

2.3.1. The clinical Data

For stage one (retrospective chart reviews) and stage two (questionnaire-based cross-section), the results were presented as mean with standard deviation (SD) for normally distributed data, median with interquartile range (IQR) for skewed results and frequency (number and percentage) of participants as appropriate. For categorical variables, groups were compared using chi-square test, and the Fisher's exact test (two-tailed) replaced the chi-square in case of small sample size, where the expected frequency is less than 5 in any of the cells. Pearson's and Spearman's correlation coefficients were calculated to assess the correlation between parametric and non-parametric variables respectively. Data analysis was performed using Statistical Package for Social Sciences (SPSS Chicago IL, USA) for windows version 25. The level of statistical significance was set at $P<0.05$

2.3.2. Microbiome analysis

Microbiome analysis was conducted using Axiom™ MiDAS, which is an analysis package for automated high-resolution genomic detection of microbial samples, designed for use with data generated from Axiom Microbiome Arrays. Axiom MiDAS performs single-sample analysis of CEL files from Axiom Microbiome Arrays and automatically generates a comprehensive analysis summary in a simple-to-use software package. High-resolution genomic identification of archaea, bacteria, fungi, protozoa, and viruses can be detected in complex microbial samples.
Adapted from ThermoFisher Scientific website; accessed 1/1/2019
(https://www.thermofisher.com/qa/en/home/life-science/microarray-analysis/microarray-analysis-instruments-software-services/microarray-analysis-software/axiom-microbial-detection-analysis-software.html)

For establishing the alpha diversity: We utilised R Package (microbiomesick) for microbiome:https://www.bioconductor.org/packages/release/bioc/html/phyloseq.html

For establishing the beta diversity: We utilized R Package (betapart) for microbiome: https://cran.r-project.org/web/packages/betapart/betapart.pdf

2.3.3. microRNA data analysis

Transcriptome Analysis Console (TAC) Software was the program utilised to run miRNA analysis following running the samples on the microarray's plates. The software includes the functionality of Expression Console (EC) Software, and allows array QC and data normalisation to be performed, along with statistical tests for differential expression, and focusing on genes or pathways of interest, exploring interactions between coding and non-coding RNA, interpreting complex alternative splicing events, linking out to publicly available annotations, and obtaining sequence information to design validation experiments.

Figure 12: The analysis of miRNA by utilising TAC Software technology.

TAC software that was used to perform results normalisation and statistical tests for the different expression of miRNA.

Adapted from ThermoFisher Scientific website; accessed 1/4/2019 https://www.thermofisher.com/qa/en/home/life-science/microarray-analysis/microarray-analysis-instruments-software-services/microarray-analysis-software/affymetrix-transcriptome-analysis-console-software.html.

CHAPTER 3

RESULTS. CLINICAL STRATIFICATION OF PATIENTS WITH PIDD

3.1. Introduction

This is the first of two results chapters. This chapter summarises data related to the retrospective data collection and analysis of the patients of PIDD seen at Hamad Medical Corporation, Adult Allergy and Immunology Unit (stage 1) and results of the questionnaire-based cross-sectional study that was used to select patients for the intervention step of the study (stage 2) (Figure 7).

The most recent classification of PIDDs was published in January 2018, by the International Union of Immunological Societies (IUIS) PID expert committee (EC), on Primary Immunodeficiency. The committee meets and publishes every other year a classification of the inborn errors of immunity (Formerly called PIDD)[3]. The IUIS divides the disorder into nine categories. The majority of patients were diagnosed in late adolescence to adulthood, as compared to those diagnosed in early life, and who have more severe monogenic forms, belong to the predominantly antibody deficiency (PAD) category (common variable immunodeficiency (CVID), immunoglobulin A deficiency (SIgAD), Specific antibody deficiency (SAD) and IgG subclass deficiency)[90-91]. The geographical location and ethnicity play a role in determining the prevalence and clinical characteristics of PIDD based on epidemiological studies and international registries. Factors such as parent consanguinity and dietary habits are pertinent to PIDD studies and so must always be evaluated. PIDD in the MENA countries was associated with the higher consanguinity in Arabic countries[28].

The complications of the PIDDs received particular attention, especially in CVID, to evaluate the different disease phenotypes, outcomes and survival rates. PIDD is associated with multiple complications like chronic or recurrent infections, inflammatory conditions, and a variety of haematological and autoimmune diseases. Immune thrombocytopenic purpura (ITP), and autoimmune haemolytic anaemia (AHA) are the most common with a frequency of 7.6% and 4.8% respectively in CVID. Also, cytopenia was found common in congenital immune deficiency as well as in antibody defects including CVID and SIgAD. Different mechanisms have been proposed to explain this association like the presence of defects in T-cell regulation, cytokine defects, abnormal apoptosis, and the abnormal production of immunoglobulins with autoimmune features[92]. Other reports stated that the respiratory system is the most frequent site of the clinical manifestations in PID and has the highest morbidity and mortality. Recurrent respiratory infections are often the first warning sign and may lead to death in up 45% of cases. The types of complications associated with the respiratory system include recurrent, severe, and persistent infections (sinuses, ears,

throat and lungs) and with opportunistic or unusual pathogens. Also, immune dysregulation (in the form of autoimmunity, allergy, and lymphoproliferative disorders), structural abnormalities like bronchiectasis, interstitial lung diseases, organising pneumonia, and pulmonary adenopathies, and malignancies. Hyper-reactive airway diseases is another consideration particularly in CVID and Selective IgA Deficiency (SIgAD) (15-45% of patients). These patients may present or have a concomitant history of asthma and other types of atopic disorders and the diagnosis is challenging. Finally, pulmonary complications of PIDD treatment with IGIV and/or transplantation are iatrogenic injuries to be added to the list of complications[93].

3.2. Aims of the study

1. To evaluate the clinical and immunological phenotypes of PIDD patients in an adult immunology service and to evaluate the morbidity related to disease complications in order to reach a better clinical stratification of patients with PIDD.
2. To investigate clinical and chemical biomarkers in the presence and absence of complications in patients with PIDD.

3.1. Methods

The materials and methods have been described in detail in sections 2.1.1–2.1.3, and sections 2.1.5 and 2.2.1. Briefly, two research designs were conducted, and the results are summarised in this chapter. A retrospective chart review was conducted for 48 medical records of PIDD patients followed over the previous 10 years. The second part is a questionnaire-based cross-sectional study for 26 PIDD patients in adult allergy and immunology service in the tertiary service (Hamad General Hospital) in Qatar. The study received local ethical approval and a grant. Data were collected for demographic characteristics, disease details (clinical and chemical biomarkers) and associated complications. Two questionnaires were used to evaluate patients' dietary habits and clinical stability (see Appendices III & IV). Ten patients were selected based on the questionnaires' analysis to be included for interventional part of the study (stage 3; discussed in Chapter 4).

3.2. Results: Retrospective data analysis

3.4.1. Demographic and clinical characteristics of the study subjects

The wide spectrum of immunodeficiency phenotypes were analysed based on the existing immunodeficiency defects and the IUIS classification. Forty eight patients (different

immunodeficiency disorders) registered at the adult Allergy and Immunology Unit, Hamad Medical Corporation, in Qatar over the last ten years were retrospectively analysed based on their demographic profile. There were 28 (58.3%) male and 20 (41.7%) female patients making a male: female ratio of 1.4. The age of patients ranged from 10 to 74 years, with a mean age of 24.3 years. Positive family history was observed in 21 (43.8%) patients and the parents of 7 (14.6%) patients were consanguineous. The majority of patients (n= 42, 87.5%) were of Arab ethnicity. In total, 26 (54.2%) were diagnosed in adulthood and 44 (91.7%) developed at least one of the disease-related complications (Table 6).

For simplicity, patients with PIDD were divided into two groups; Humoral or B-cell PIDD and Other PIDD. Combined T and B-cell immunodeficiency (CID), with syndromic features and Predominantly Antibody (Production) Deficiency (PAD) constitute the humoral or B-cell PIDD (Category 2 and 3 of the IUIS classification). The Other PIDD group is composed of four IUIS categories (defects in intrinsic and innate immunity, congenital defects of phagocyte number, function, or both, complement deficiencies, and diseases of immune dysregulation). Refer to Table 1 in chapter 1 for the IUIS classification. The most prevalent disorders were CVID (n = 9, 18.8%), SAD (n = 7, 14.6%), and XLA (n = 6, 12.5%) and hypogammaglobulinaemia (n = 6, 12.5%). Combining XLA and hypogammaglobulinaemia may give a higher estimated rate for this particular group of patients with very similar diagnosis 12 (25%). Among the 31 patients with PAD, CVID accounted for 9 (29%), SAD (n = 7, 22.6%), and XLA (n = 6, 19.4%) and hypogammaglobulinaemia 6 (19.4%). In addition, combining XLA and hypogammaglobulinaemia may give a higher estimated rate for this particular group of patients with very similar diagnosis (n = 12, 38.7%). One case of complement system deficiency (C7 complement deficiency) is recorded in this cohort. Tables 7 and 8 describe the different diseases and patient demographics.

Characteristics	B-cell PIDD n= 36	Other PIDD n=12	All PIDD n= 48	*P*-value
Age in years (mean ± SD (range))	25.94 ± 11.89 (10-74)	19.3 ± 3.55 (14-26)	24.29 ± 10.79 (10-74)	0.005
Male (actual number (%))	21 (58.3%)	7 (58.3%)	28 (58.3%)	0.999
Body Mass Index (mean ± SD (range))	23.53 ± 7.91 (10.8-44.89)	22.23 ± 4.83 (15.1-30.47)	23.03 ± 7.19 (10.8-44.89)	0.595
Ethnicity Arab (actual number (%))	31 (86.1%)	12 (100%)	42 (87.5%)	0.220
Parental consanguinity (actual number (%))	2 (5.5%)	5 (41.6%)	7 (14.6%)	0.007
Smokers (actual number (%))	4 (11.1%)	0	4 (8.4%)	0.303
Diagnosed at adult age (actual number (%))	24 (66.7%)	2 (16.7%)	26 (54.2%)	0.004
Complications (actual number (%))	36 (100%)	8 (66.7%)	44 (91.7%)	0.002

Table 6: Comparison between B-cell immunodeficiency patients and other PIDD groups.

The table PIDD. Age in years, presence of complications and presentation at adult age are statistically higher in B-cell group ($P<0.05$). Parental consanguinity was statistically higher in the other PIDD group ($P=0.007$).

No. of patients	Disease type	IUIS (Inborn error of Immunity) classification	Age in year Mean (range)	Male	Main presentation
3	AT with immunodeficiency	CID with associated or syndromic features	17.6 (15-22)	2	Recurrent infections
9	CVID	PAD	30.4 (23-43)	2	
6	XLA	PAD	23.5 (14-340	6	
1	HIES	CID with associated or syndromic features	14	1	
7	SAD	PAD	25.75 (16-42)	2	
6	Hypogammaglobulinaemia	PAD	20.5 (15-29)	5	
1	Other combined	CID with associated or syndromic features	27	1	
2	Iso. IgMD	PAD	39.3 (10-74)	0	Asthma and allergy, recurrent infection
1	SIgAD	PAD	39	1	

Table 7: Patients in the category of B-cell immunodeficiency reclassified based on IUIS.
Thirty-six patients were included. Majority of PAD is dominating in the male with male: female ratio of 1.3. The exceptions are CVID, Iso. IgMD. and SAD. The most common presentation is recurrent infection followed by atopic diseases. AT: Ataxia Telangiectasia, CVID: Common Variable Immunodeficiency, XLA: X-Linked Agammaglobulinaemia, SAD: Specific Antibody Deficiencies. Iso. IgMD: Isolated IgM deficiency, SIgAD: Selective IgA Deficiency, HIES: Hyper IgE syndrome, CID: combine immunodeficiency, and PAD: predominantly antibody deficiency.

Patient's sequence	Disease type	IUIS (Inborn error of Immunity) classification	Age	Sex	Main presentation
1	IL-12Rβ1 def.	Defects in intrinsic and innate immunity	17	Female	Recurrent infections
2	IL-12Rβ1 def.	Defects in intrinsic and innate immunity	18	Male	
3	IL-12Rβ1 def.	Defects in intrinsic and innate immunity	19	Female	
4	CGD	Congenital defects of phagocyte number, function, or both	14	Male	
5	CGD	Congenital defects of phagocyte number, function, or both	21	Male	
6	CGD	Congenital defects of phagocyte number, function, or both	18	Female	
7	ALPS post BMT	Diseases of immune dysregulation	23	Female	
8	C7 def.	Complement deficiencies	26	male	
9	Profound T cells def.	Unclassified immunodeficiency	23	Female	
10	Rab27a def.	Diseases of immune dysregulation	17	Male	Family history of siblings with same disorders
11	Rab27a def.	Diseases of immune dysregulation	21	Male	
12	Rab27a def.	Diseases of immune dysregulation	15	Male	

Table 8: Patients with other PIDD category based on IUIS classification.

Twelve patients were included. Similar to table 7, most of PIDD disorders are dominating in male patients with a male: female ratio of 7:5. Although recurrent infection is dominating, the second important presentation is family history.

IL-12Rβ1 def.: IL-12Rβ1 deficiency that leads to milder form of Mendelian susceptibility to mycobacterial disease (MSMD), CGD: chronic granulomatous diseases, Rab27 a def: *RAB27A* mutation associated with NK cell cytotoxicity deficiency, ALPS post BMT: Autoimmune lymphoproliferative syndrome post bone marrow transplantation, C7 def.: Complement C7 deficiency.

3.4.2. B-cell PIDD

3.4.2.1. PIDD Prevalence

The prevalence of different disease is calculated from the review of the data available in the adult immunology clinics of the tertiary hospital of Hamad General Hospital Allergy and Immunology service- Qatar (Figure 13). This is not a representative of prevalence of the disease across Qatar. No Severe Combined Immunodeficiency (SCID) is present in the current data.

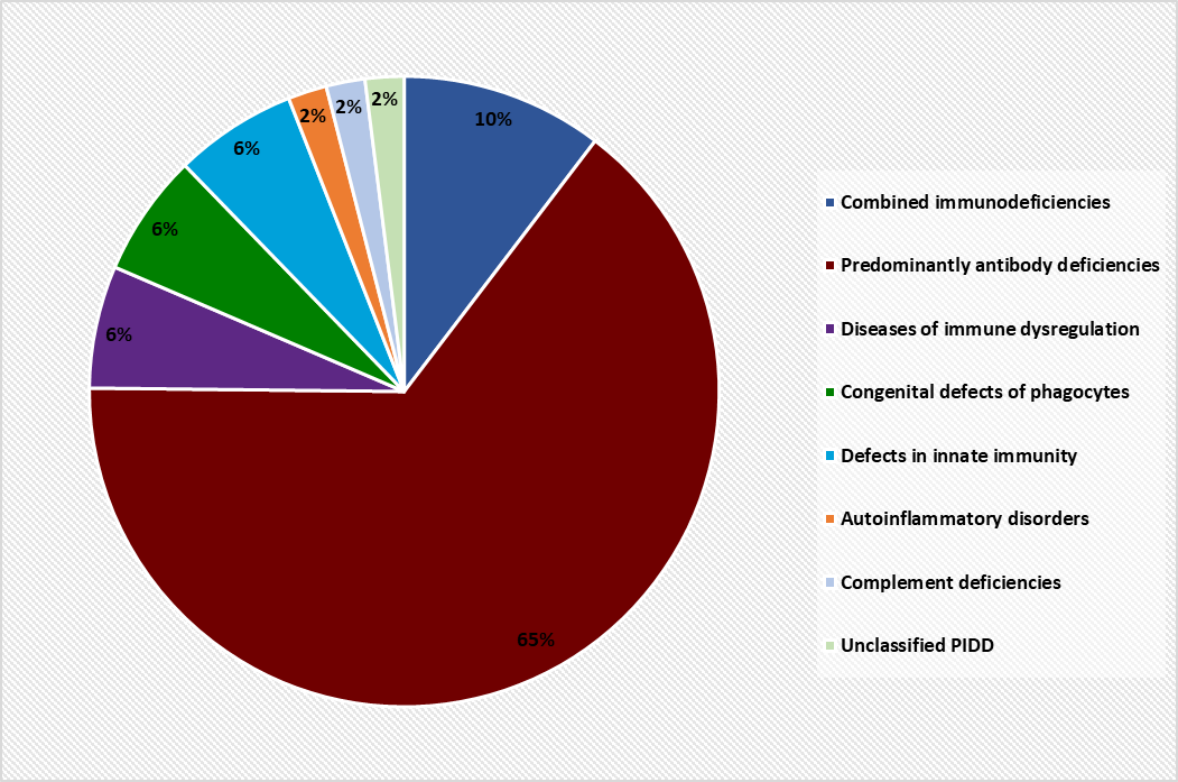


Figure 13: Relative frequency of PIDD in adult immunology clinics in a tertiary hospital in Qatar.

The distribution of the different PIDDs was similar to the global distribution. Predominantly Antibody Deficiency (PAD) was dominating, followed by Combined Immunodeficiency (CID), defects in phagocytes and diseases in immune dysregulation. The patients were followed over the last 10 years.

3.4.2.2. B-cell PIDD and complications

Among the 48 patients in this study, 36 had B-cell or humoral immunodeficiency of different diseases subtype and different complications distribution. The most common complications, which were predominated amongst the 36 patients with mainly B-cell immunodeficiency, were recurrent infections (n= 36, 100%), bronchiectasis (n= 26, 29%) and allergy (n= 8, 9%). The highest rate of complications was observed in CVID (n= 24, 26.7%), SAD (n= 19, 21%), XLA (n= 13, 14.4%) and hypogammaglobulinaemia (n= 11, 12.2%). Moreover, the recurrent infections and bronchiectasis were dominating in the PAD commonest diseases (CVID and SAD). The allergy complication was dominating in the isolated IgM deficiency (Iso. IgMD) and SAD while sparing the CVID. Non-infectious complications including autoimmunity and auto-inflammatory complications were present in patients with CVID and SAD and were spared in XLA and other hypogammaglobulinaemia patients. Auto-inflammatory complications in this cohort referred to lymphoproliferative and inflammatory complications that involve the gut (enteropathy), liver, lungs (intestinal lung disease and granuloma), and lymphatic hyperplasia. Malignancy is one of the most severe outcomes of the diseases and was diagnosed in three patients. One of these patients presented with Non-Hodgkin's lymphoma (NHL), and bronchiectasis with a history of recurrent infections and severely low immunoglobulins. The patient was later diagnosed with CVID. The other two patients also had malignancies (leukaemia and lymphoma) and died during this study. The mortality was not present at the time of collection of these data. However, three patients died during the next stages of this research study; two were due to malignancies (mentioned above) and the third was due to vasculopathy and bleeding disorder (Table 9 and Figure 14).

Characteristic	Disease class based on 2017 IUIS phenotypic classification									
	Class II: CID n= 5			Class III: PAD. n= 31						
Disease Name	AT	HIES	Other combined	CVID	XLA	SAD	Hypo-gamma	SIgAD	Iso. IgMD	Total number
Number of patients	3	1	1	9	6	7	6	1	2	36
Recurrent infection	3	1	1	9	6	7	6	1	2	36
Bronchiectasis	3	0	0	9	6	5	3	0	0	26
Allergic diseases	0	1	0	0	0	3	1	1	2	8
Auto-inflammatory	0	0	0	2	0	2	0	0	1	5
Autoimmunity	0	0	0	2	0	2	0	0	0	4
Malignancy	1	0	1	1	0	0	0	0	0	3
Endocrinopathy	0	0	0	0	1	0	0	0	2	3
arthritis	1	0	0	1	0	0	0	0	0	2
Death	1		1				1			3
Total number of complications	9	2	3	24	13	19	11	2	7	90

Table 9: Distribution of complications.

Thirty-six patients with B-cell immunodeficiency were followed in Hamad Medical Centre, based on IUIS classification.

The most common complication is recurrent infection, bronchiectasis and allergy. Autoimmunity include immune thrombocytopenic purpura, autoimmune haemolytic anaemia and autoimmune neutropenia. The three patients with malignancy: one with Leukaemia and two with lymphoma. Endocrinopathy includes thyroid disorders and diabetes mellitus. Auto-inflammatory includes lymphoproliferative and inflammatory complications that involve gut (enteropathy), liver, lungs (intestinal lung disease and granuloma), and lymphatic hyperplasia. AT: Ataxia Telangiectasia, CVID: Common Variable Immunodeficiency, XLA: X-Linked Agammaglobulinaemia, SAD: Specific Antibody Deficiencies. IgMD: Isolated IgM deficiency, SIgAD: Isolated IgA deficiency, HIES: Hyper IgE syndrome.

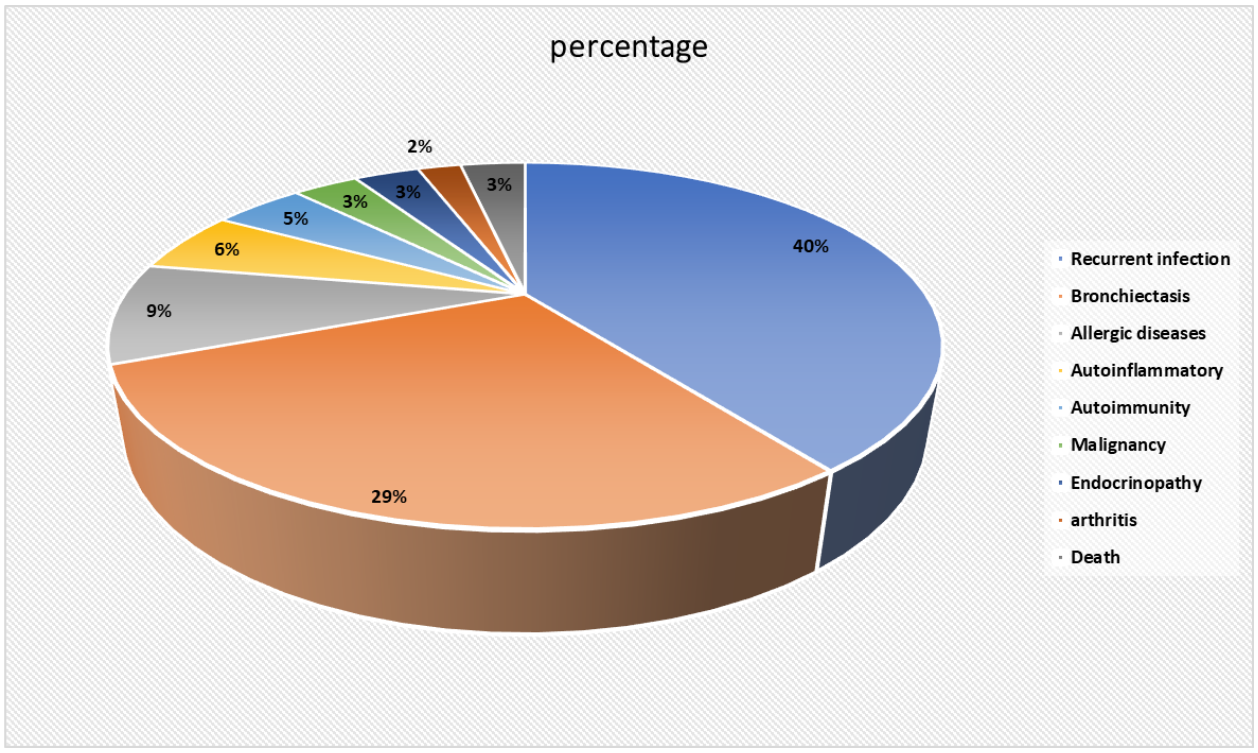


Figure 14: Percentage distribution of complications.

This is based on complications (total number of complications=81).

3.4.3. The correlation between clinical parameters in B-cell PIDD and the commonest complications

Comparison is made between the different clinical parameters including IUIS classification (CID vs. PAD), parent consanguinity, ethnicity (Arabs vs. others), gender (male vs. female), smoking (smokers vs. non-smokers) and the age at which the diagnosis was first made (paediatric vs. adult) in the complications of bronchiectasis and allergy. (Figure 15 and 16). Age of presentation to health service was not a reliable indicator of the presence of multiple comorbidity or severe outcome. However, patients who presented in paediatric age group (<14 years old) had greater incidence of bronchiectasis in 11 out of 12 (91.7%) compared to those diagnosed at adult age 9 out of 24 (37.5%) (P=.002). Gender and smoking were not associated with an increased rate of overall complications (P>.05). However, males were more likely to develop bronchiectasis than females (P=.003). Smoking, ethnicity and parent consanguinity were not associated with increased rate of bronchiectasis complications in this cohort (P>.05). For the complication of allergy, only the age of presentation was associated with statistically significant value. Patient diagnosed at adult age (≥ 14), were more likely to develop allergic disorders.

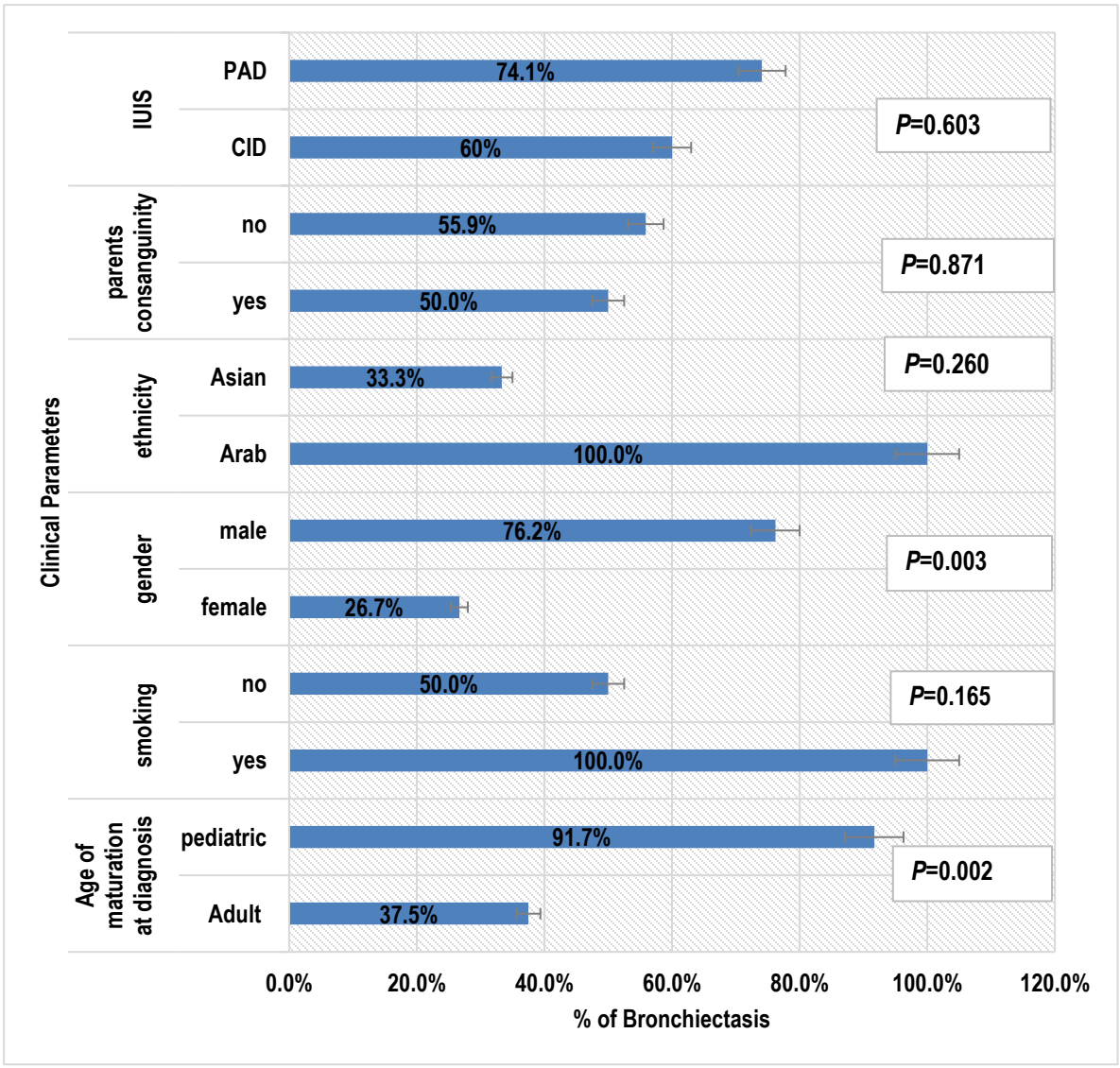


Figure 15: The different clinical parameters in the complication of bronchiectasis.

The complication of bronchiectasis is more represented among males and those diagnosed at paediatric age ($P<0.05$)

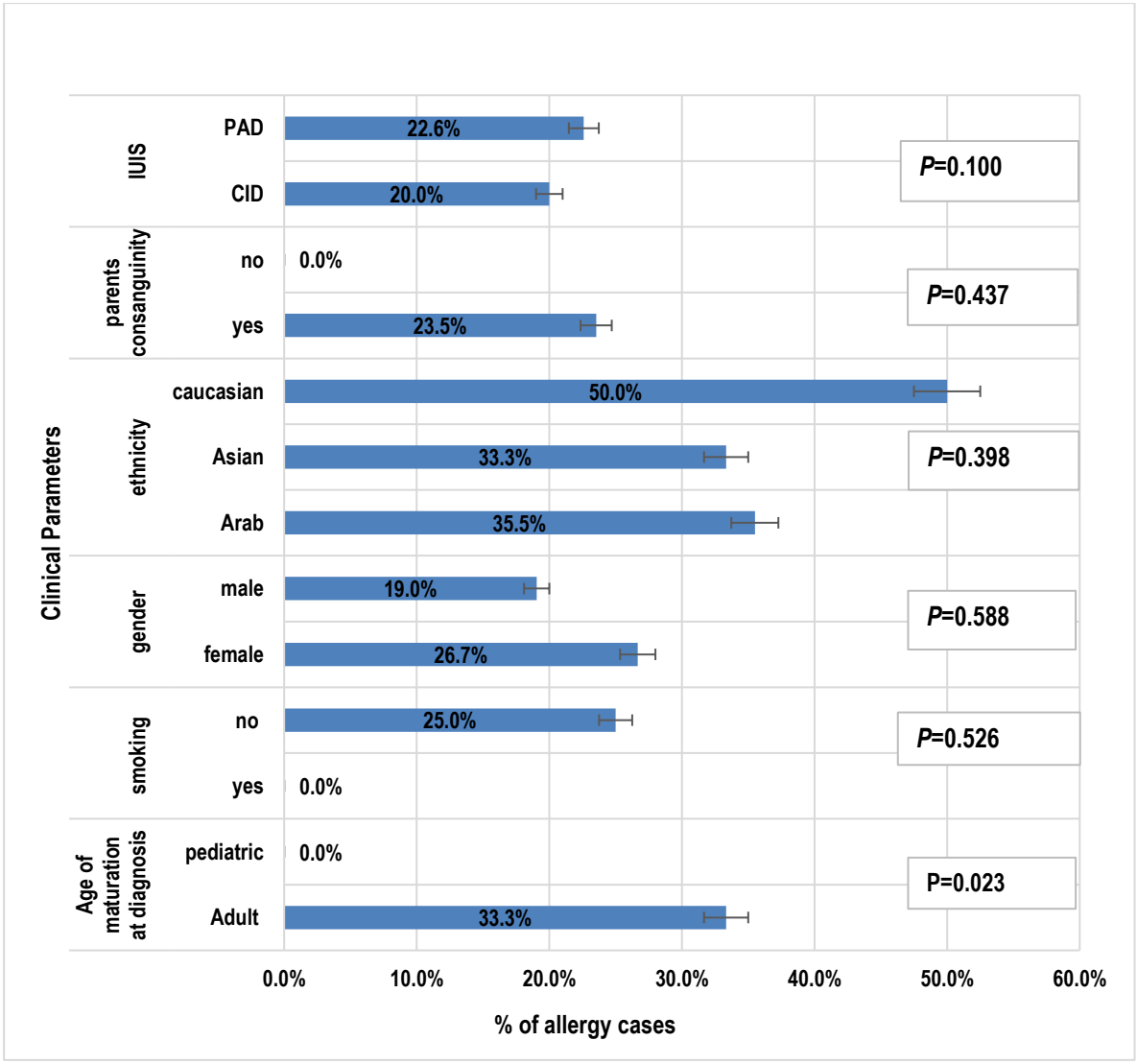


Figure 16: The different clinical parameters in the complication of allergy.

The complication of Allergy is statistically higher among those diagnosed at adult age ($P<0.05$).

3.4.4. The correlation between the chemical biomarkers in patients with B-cell PIDD and the two complications of bronchiectasis and allergy

The mean and median were computed for the chemical biomarkers in each one of the most common complications (bronchiectasis and allergy), to identify possible correlations. Tables 10 and 11 summarise selected chemical biomarkers that are of clinical significance in many medical conditions with inflammatory and autoimmune complications. Those biomarkers were compared in the patients with PIDD who have complications to those who have not.

Vitamin D was low in most of the patients (33 out of 36). However, the difference between patients with PIDD in both bronchiectasis and allergy complications compared to those with no complications for the vitamin D deficiency was not statistically significant ($P>0.05$). CRP and initial IgG level were statistically significant in the bronchiectasis and allergy complications groups ($P<0.05$). Mean and median of White Blood Cells (WBC) was statistically higher in patients without complications ($P=0.033$). Tables 10, 11a and 11b.

Vit D	Initial IgG level @ diagnosis	CD3 (count &%)
Vitamin B12	IgA	CD4 (count &%)
S. folate	IgM	CD8 (count &%)
RBC folate	IgG1	CD19 (count &%)
Vitamin A	IgG2	CD3-, CD16+, CD56+ (count &%)
Vitamin E	IgG3	CD4/CD8 ratio
WBC	IgG4	Glucose
Hgb	TIgE	HgA1C
Plt	IgG level (last)	TSH
Lymphocytes	CRP	C3
Neutrophils (count&%)	ESR	C4
Eosinophil (count&%)	Ferritin	AFP

Table 10: Chemical and blood biomarkers as per medical records.

Forty-eight patients were included in the retrospective study. WBC: White blood test, Hgb: Haemoglobin, Plt: platelets, Ig; Immunoglobulin, TIgE: Total IgE, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, CD: cluster of differentiation (Lymphocytes cell co receptors and markers), HbA1C: Haemoglobin A1C (i.e. average levels of blood glucose over the last 3 months), TSH: Thyroid-stimulating hormone, C3: complement 3, C4: complement 4, AFP: α-fetoprotein.

Chemical Biomarkers (units)	B-cell PIDD				
	bronchiectasis		No bronchiectasis		*P*-value
	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
Vitamin D (ng/ml)	16.3 (7.6)	17.0 (10.0-21.0)	21.3 (14.3)	17.0 (12.0-26)	0.082
Vitamin B12 (pmol/L)	510.1 (385.0)	409.0 (219.3-902.0)	265.4 (124.7)	208.5 (179.5-353)	0.402
CRP (mg/L)	37.6 (50.2)	15.5 (6.5-67.1)	9.4 (14.0)	5.0 (3.3-8.2)	0.045
ESR (mm/hr)	20.1 (19.3)	14.5 (5.3-27.8)	16.4 (18.1)	11.5 (2.0-20.3)	0.374
Ferritin (mcg/L)	61.9 (38.8)	54.0 (26.8-102.5)	47.6 (41.2)	43.3 (7.0-74.8)	0.664
WBC ($x10^3$/ul)	8.4 (3.5)	8.3 (5.8-9.2)	23.5 (68.2)	6.3 (3.9-8.8)	0.033
Lymphocytes%	23.4 (16.1)	24.9 (6.9-34.4)	24.1 (16.5)	32.2 (5.5-36)	0.361
HbA1C%	5.3 (0.5)	5.6 (4.8-5.7)	5.5 (1.5)	5.2 (4.8-5.3)	0.742
TSH (mIU/L)	3.0 (2.6)	1.9 (1.3-3.7)	3.1 (5.4)	1.7 (1.1-2.3)	0.832
Initial IgG (mg/dL)	251.1 (332.9)	100.0 (100.0-191.5)	801.5 (630.0)	732.0 (286.5-1270)	0.022

Table 11a: The different chemical biomarkers in PIDD patients with the complication of bronchiectasis.

Vitamins and acute phase reactants (CRP, ESR and Ferritin) were evaluated and compared in patient with PIDD with and without bronchiectasis complication.

CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, WBC: White blood test, HbA1C: Haemoglobin A1C (i.e. average levels of blood glucose over the last 3 months), TSH: Thyroid-stimulating hormone, Ig: Immunoglobulin

Chemical Biomarkers (units)	B-cell PIDD				
	Allergy		No allergy		*P*-value
	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
Vitamin D (ng/ml)	23.1 (19.3)	15.5 (10.3-36.0)	17.1 (7.2)	17.0 (12.0-22.0)	0.623
Vitamin B12 (pmol/L)	237.3 (139.0)	201.0 (146.0-255.0)	449.4 (343.5)	338.0 (196.5-463.0)	0.236
CRP (mg/L)	8.0 (5.1)	5.0 (5.0-15.0)	30.1 (45.1)	10.0 (5.0-38.0)	0.010
ESR (mm/hr)	14.9 (22.3)	3.0 (2.0-24.0)	19.4 (17.6)	16.0 (9.0-24.0)	0.120
Ferritin (mcg/L)	34.4 (31.7)	24.5 (5.8-72.3)	61.5 (40.9)	58.8 (26.3-99.0)	1.000
WBC ($x10^3$/ul)	40.8 (96.3)	8.6 (3.6-10.5)	7.8 (3.5)	6.7 (5.6-8.9)	0.543
Lymphocytes%	18.5 (21.2)	6.2 (1.8-44.0)	25.2 (14.4)	28.5 (12.0-34.9)	0.580
HbA1C%	5.8 (1.9)	5.0 (4.8-5.8)	5.3 (0.5)	5.3 (4.9-5.6)	0.964
TSH (mIU/L)	4.9 (7.6)	1.7 (1.1-7.2)	2.4 (1.9)	2.0 (1.1-3.0)	0.628
Initial IgG (mg/dL)	781.6 (957.4)	300.0 (59.0-1745.0)	431.2 (437.3)	183.0 (100.0-786.0)	0.001

Table 11b: The different chemical biomarkers in PIDD patients with the complication of allergy.

Vitamins and acute phase reactants (CRP, ESR and Ferritin) and other parameters were evaluated and compared in patient with PIDD with and without Allergy complication.

CRP: C-Reactive Protein, ESR, WBC: White blood test, HbA1C: Haemoglobin A1C (i.e. average levels of blood glucose over the last 3 months), TSH: Thyroid-stimulating hormone, IgG: Immunoglobulin G

3.5. Results: Analysis of the two questionnaires

Forty patients were enrolled onto the cross-sectional questionnaires study (stage 2). Two patients were withdrawn due to ineligibility (on nasogastric tube feeding), ten did not complete all components of the questionnaires and were removed, and two patients were excluded because of implausible serving intake. The cohort of 26 patients were included in the final analysis.

3.5.1. Dietary questionnaire analysis

Analysis of the dietary questionnaire was used to evaluate the servings of each food item per day and to identify correlation to the demographics of the patients (age and BMI). Also, it was done to evaluate dietary habits prior to intervention with vitamins.

3.5.1.1. Demographic characteristics of diet intake of the patients with B-cell PIDD

There was almost equal sex distribution with a mean age of 27.9. The mean BMI was 26.2. Most of the patients were taking enough servings of fruits (4.4) and vegetables (6.2). Those with extreme outliers were not selected for the intervention study. Table 12

Characteristics		**N=26**
AGE (Mean +/- SD (range)) years		27.88 +/- 11.68 (15-60)
Female frequency (%)		14 (53.8%)
BMI (Mean +/- SD (range)) kg/m^2		26.23 ± 8.94 (10.80-44.89)
Food items per day (based on an average 2,000 calorie **per day** diet)	Dairy (Mean +/- SD (range)) (servings/day)	2.43 ± 2.34 (0-11.29)
	Fruit (Mean +/- SD (range)) (servings/day)	4.12 ± 4.41 (0-17.98)
	Vegetables (Mean +/- SD (range)) (servings/day)	6.16 ± 3.26 (1.58-11.24)
	Cereals/seeds (Mean +/- SD (range)) (servings/day)	2.92 ± 2.78 (0.22-13.01)
	Legumes/Nuts (Mean +/- SD (range)) (servings/day)	0.66 ± 0.98 (0-3.75
	Meat (Mean +/- SD (range)) (servings/day)	2.26 ± 1.25 (0.08-4.93)
	Snacks (Mean +/- SD (range)) (servings/day)	0.94 ± 0.97 (0-4.08)
	Sweets (Mean +/- SD (range)) (servings/day)	1.09 ± 1.43 (0-7.24)
	Beverages/Alcohol (Mean +/- SD (range)) (servings/day)	1.59 ± 1.78 (0-6.0)

Table 12: Analysis of the dietary questionnaire.

Most patients receive good servings of vegetables and fruits with a variable intake of sweets and cereals meals.

3.5.1.2. Correlation between dietary intake, age, and BMI

Spearman's rho correlation coefficient was used to assess the relationships between age, BMI and dietary intake (Table 13).

Spearman's correlation analysis between age, BMI and the different dietary classes scores showed a negative correlation for sweet intake ($r = -0.438$ $P<0.05$).

The sweets were negatively correlated to BMI. The Mean of the servings of sweet was 1 (range from 0-7; Table 12) indicating variable intake of sweets in the current cohort. Also, in Table 12, the intake of cereals meals was also of variable degree and can reach up to 13 servings. However, it has no correlation with BMI.

Dietary class	AGE		BMI	
	r	*P*-value	r	*P*-value
Dairy	-0.012	0.955	-0.247	0.224
Fruit	-0.378	0.057	-0.177	0.387
Vegetables	-0.228	0.263	-0.109	0.596
Cereals/seeds	-0.213	0.295	-0.032	0.875
Legumes/Nuts	-0.294	0.145	-0.213	0.296
Meat	-0.324	0.106	-0.191	0.35
Snacks	-0.228	0.263	-0.354	0.076
Sweets	-0.331	0.099	-0.438*	0.025
Beverages/Alcohol	0.258	0.204	0.12	0.558

*. Correlation is significant at the *P*<0.05 level (2-tailed).

**. Correlation is significant at the *P*<0.01 level (2-tailed).

Table 13: Correlation between dietary intake, age and BMI in patients with B-cell PIDD.

Non-parametric correlation using Spearman's rho correlation coefficient.

3.5.1. Symptom questionnaire analysis

The analysis of the symptom questionnaires helped to select clinically stable patients and exclude patients whose physical and mental health may interfere with the participation in the interventional part of the study (stage 3). A correlation analysis was performed between the symptoms and the patients' demographics (age, sex and BMI) (Tables 12-17).

3.5.2.1. Descriptive analysis of the general symptoms

The most common symptoms were Compulsive eating (n = 25, 96.2%), discoloured lips (n = 25, 96.2%), lack of energy with feeling of fatigue/sluggishness (n = 16, 61.5%), and cough (n = 13, 50%) (Table 14).

Symptoms (n=26)		Frequency n (%)
DIGESTIVE TRACT	Nausea or Vomiting	6 (23.1%)
	Diarrhoea/ constipation	8 (30.7%)
	bloating feeling	11 (42.3%)
	Heart burn	7 (26.9%)
	Intestinal/stomach pain	7 (26.9%)
EARS	Itchy ears	4 (15.4%)
	Earaches	2 (7.7%)
	Drainage from ear	3 (11.5%)
EMOTIONS	Mood swings	11 (42.3%)
	Anxiety, fear or nervousness	5 (19.2%)
	Anger, irritability or aggressiveness	4 (15.4%)
	Depression	2 (7.7%)
ENERGY/ ACTIVITY	Fatigue, sluggishness	16 (61.5%)
	apathy, lethargy	2 (7.7%)
	hyperactivity	2 (7.7%)
	Restlessness	5 (19.2%)
EYES	Watery or itchy eyes	9 (34.6%)
	Swollen, reddened or sticky eye lids	2 (7.7%)
	Blurred or tunnel vision	3 (11.5%)
HEAD	Head ache	10 (38.5%)
	Faintness	2 (7.7%)
	Insomnia	3 (11.5%)
HEART	Irregular or skipped heartbeat	2 (7.7%)
	Chest pain	5 (19.2%)
JOINTS/MUSCLES	Pain or aches in joints	10 (38.5%)
	Arthritis	3 (11.5%)
	Pain or aches in muscles	7 (26.9%)
	Feeling of weakness or tired	7 (26.9%)
CHEST	Difficulty in breathing	5 (19.2%)
	Cough	13 (50.0%)
MIND	Poor memory	3 (11.5%)
	Confusion, poor comprehension	3 (11.5%)
	Difficulty in making decision	4 (15.4%)
	Learning disabilities	2 (7.7%)
NOSE	Stuffy nose	8 (30.8%)
	Sinus problem	9 (34.6%)
	Sneezing attacks	4 (15.4%)
SKIN	Swollen/discoloured tongue, gum or lips	25 (96.2%)
	Hives, rashes or dry skin	5 (19.2%)
	Hair loss	12 (46.2%)
WEIGHT	Binge eating/drinking	3 (11.5%)
	Excessive weight	5 (19.2%)
	Compulsive eating	25 (96.2%)
	Under weight	5 (19.2%)
OTHER	Frequent illness	5 (19.2%)
	Genital itch or discharge	2 (7.7%)

Table 14: Frequency of common symptoms in patients with PIDD.

Feelings of fatigue, depression, coughing and compulsive eating were the most common symptoms.

3.5.2.2. The Well-being Questionnaires

These questionnaires were adapted from SF-6D[86] and GHQ12[87] to evaluate the patients' general health and mental health prior to enrolment in the interventional part of the study. We were able to select 10 of the 26 patients, evaluated by these questionnaires, for stage 3 (intervention with vitamins) of the study. Those with functional limitations or mode instability, based on these questionnaires results, were excluded from the study selection for the intervention stage. Stage 3 of the study and the analysis of these 10 patients is explored in chapter 4.

Among the 26 patients, 23 patients had god quality of life with minimal pain or limitation in daily physical activities based on SF-6D quality of life questionnaire. Moreover, GHQ12 mental questionnaire revealed normal mental status in almost all patients with no evidence of depression symptoms (Tables 15 and 16).

SF-6D (Quality of life Questionnaire)

Symptoms (n=26)		Frequency n (%)
PHYSICAL FUNCTIONING (PF)	Your health does not limit you in vigorous activities	12 (46.2%)
	Your health limits you a little in vigorous activities	12 (46.2%)
	Your health limits you a little in moderate activities	2 (7.7%)
	Your health limits you a lot in moderate activities	1 (3.8%)
	Your health limits you a little in bathing and dressing	3 (11.5%)
	Your health limits you a lot in bathing and dressing	1 (3.8%)
ROLE LIMITATION (RL)	You have no problems with your work or other regular daily activities as a result of your physical health or any emotional problems	18 (69.2%)
	You are limited in the kind of work or other activities as a result of your physical health	3 (11.5%)
	You accomplish less than you would like as a result of emotional problems	2 (7.7%)
	You are limited in the kind of work or other activities as a result of your physical health and accomplish less than you would like as a result of your emotional problems	1 (3.8%)
SOCIAL FUNCTIONING (SF)	Your health limits your social activities none of the time	15 (57.7%)
	Your health limits your social activities a little of the time	7 (26.9%)
	Your health limits your social activities some of the time	1 (3.8%)
	Your health limits your social activities most of the time	3 (11.5%)
	Your health limits your social activities all of the time	0 (0%)
PAIN (P)	You have no pain	9 (34.6%)
	You have pain, but it does not interfere with your normal work (both outside the home and house work)	8 (30.8%)
	You have pain, that interfere with your normal work (both outside the home and house work) a little bit	5 (19.2%)
	You have pain, that interfere with your normal work (both outside the home and house work moderately	3 (11.5%)
	You have pain, that interfere with your normal work (both outside the home and house work quite a bit	0 (0%)
	You have pain, that interfere with your normal work (both outside the home and house work extremely	0 (0%)
MENTAL HEALTH (MH)	You feel tense or downhearted and low none of the time	13 (50%)
	You feel tense or downhearted and low a little of the time	7 (26.9%)
	You feel tense or downhearted and low some of the time	2 (7.7%)
	You feel tense or downhearted and low most of the time	1 (3.8%)
	You feel tense or downhearted and low all of the time	0 (0%)
VITALITY (V)	You have a lot of energy all of the time	5 (19.2%)
	You have a lot of energy most of the time	9 (34.6%)
	You have a lot of energy some of the time	10 (38.5%)
	You have a lot of energy a little of the time	1 (3.8%)
	You have a lot of energy none of the time	1 (3.8%)

Table 15: Analysis of symptoms in patients with PIDD based on SF-6D Questionnaire.

The majority of patients with PIDD in this cohort were in good well-being and no current symptoms.

GHQ-12 (Mental Health)

Symptoms (Have you recently) (n=26)		Frequency n (%)
1. Been able to concentrate on what you are doing?	Better than usual	6 (23.1%)
	same as usual	17 (65.4%)
	less than usual	1 (3.8%)
	much less than usual	2 (7.7%)
2. Lost much sleep over worry?	Not at all	12 (46.2%)
	No more than usual	10 (38.5%)
	Rather more than usual	2 (7.7%)
	Much more than usual	2 (7.7%)
3. Felt you were playing a useful part in things?	More so than usual	4 (15.4%)
	Same as usual	19 (73.1%)
	less useful than usual	1 (3.8%)
	Much less useful	1 (3.8%)
4. Felt capable of making decisions about things?	more so than usual	6 (23.1%)
	same as usual	17 (65.4%)
	Less useful than usual	2 (7.7%)
	much less capable	1 (3.8%)
5. Felt constantly under strain?	Not at all	9 (34.6%)
	No more than usual	13 (50%)
	Rather more than usual	2 (7.7%)
	Much more than usual	1 (3.8%)
6. Felt you could not overcome your difficulties?	Not at all	13 (50%)
	No more than usual	6 (23.1%)
	Rather more than usual	3 (11.5%)
	Much more than usual	1 (3.8%)
7. Been able to enjoy your 2rmal day-to day activities?	More so than usual	3 (11.5%)
	Same as usual	19 (73.1%)
	Less so than usual	3 (11.5%)
	Much less than usual	1 (3.8%)
8. Been able to face up to your problems?	More so than usual	3 (11.5%)
	Same as usual	19 (73.1%)
	Less so than usual	3 (11.5%)
	Much less able	1 (3.8%)
9. Been feeling unhappy and depressed?	Not at all	13 (50%)
	No more than usual	9 (34.6%)
	Rather more than usual	4 (15.4%)
	Much more than usual	0 (0%)
10. Been losing confidence in yourself?	Not at all	18 (69.2%)
	No more than usual	5 (19.2%)
	Rather more than usual	1 (3.8%)
	Much more than usual	0 (0%)
11. Been thinking of yourself as a worthless person?	Not at all	23 (88.5%)
	No more than usual	1 (3.8%)
	Rather more than usual	1 (3.8%)
	Much more than usual	0 (0%)
12. Been feeling reasonably happy, all things considered?	More so than usual	3 (11.5%)
	About same as usual	20 (76.9%)
	Less so than usual	2 (7.7%)
	Much less than usual	0 (0%)

Table 16: Analysis of symptoms in patients with PIDD based on GHQ12 Questionnaire.

The majority of patients expressed normal mental health.

3.5.2.3 Correlation between symptoms questionnaire and the age, gender and BMI

Pearson's correlation coefficients were used to assess the relationship between Age, gender and BMI to symptoms (Table 17).

Pearson's correlation analysis between age, gender, and BMI and the different symptoms scores showed a negative correlation for the faintness, hair loss, perception of excessive weight, and frequent urination and a positive correlation with difficulty in making decision, and frequent illnesses (Table 17).

Spearman's rho (n=25)						
symptoms	AGE		Gender		BMI	
	r	*P*-value	r	*P*-value	r	*P*-value
Faintness	-0.041	0.846	-0.307	0.136	-0.409*	0.042
Difficulty in making decision	0.409*	0.042	-0.017	0.934	0.537**	0.006
Slurred speech	0.523**	0.007	-0.236	0.256	0.416*	0.039
Hair loss	-0.247	0.244	0.251	0.237	-0.446*	0.029
Binge eating/drinking	0.453*	0.023	-0.384	0.058	0	1
Excessive weight	-0.035	0.869	-0.32	0.119	-0.402*	0.046
Frequent illness	0.132	0.53	0.08	0.704	0.499*	0.011
Frequent or urgent urination	-0.041	0.846	-0.307	0.136	-0.409*	0.042
Genital itch or discharge	0.430*	0.032	-0.307	0.136	0.133	0.526

*. Correlation is significant at the *P*<0.05 level (2-tailed).

**. Correlation is significant at the *P*<0.01 level (2-tailed).

Table 17: Correlation between the symptoms and age, gender and BMI in patients with B-cell PIDD.

Analysis using Pearson's correlation (Only positively correlated symptoms were presented in this table). Symptoms such as neurological functions, excess weight, autoimmune hair loss and urinary tract infections showed a statistical correlation with the age and BMI (*P*<0.05). However, gender was not significantly correlated with any symptoms.

3.6. Discussion

This chapter presented the retrospective data collected from the 48 patients of PIDD seen in Hamad General Hospital, Allergy and Immunology service that were followed for the last 10 years with focus on the aetiology and frequency of PIDD and associated complications. Analysis of the two questionnaires (the dietary and symptoms and well-being) conducted as a cross-sectional stage and the sub analysis of the selected ten patients with predominantly antibody deficiency that were recruited for the study in stage 3 as also presented briefly.

The PIDD different diseases prevalence data was compared to the global prevalence estimated[12] (Chapter 1 Figure 1a), and found similarity in the frequency of PAD. The PAD has higher survival chances to adult age compared to other PIDD phenotypes. Moreover, the initial presentation of this specific category can be only in the adult age in some cases. These data support the recognition that PIDD is not only a paediatric disease. The change in life expectancy, improving health care, and the presence of milder phenotype variants that can survive to adult age with fewer disease affections and are therefore undiagnosed in childhood may explain why these patients presented in adult age. Also, the usual presentation of recurrent infections, autoimmunity and malignancy in the paediatric age group can be the focus of the treating physicians while the underlying PIDD was inconspicuous[90]. It is estimated that 25% to 40% of all types of PIDD are diagnosed in adult age[91].

Our results showed that the most common disease was PAD (n=31, 64.6%) which is comparable to those seen in the adult immunology service. SIgAD is low in our report (n=1, 2.1%), which is in contrast to that reported for various European studies (up to 50%[94]). However, similar rates were observed in Iran[94], a country that is geographically close to Qatar and may therefore share a closer ethnic background. Amongst patients with Primary Antibody Deficiency, CVID, SAD and combined XLA and hypogammaglobulinaemia account for (n=9, 29%), (n=7, 22.6%), and (n=12, 38.7%) respectively in our data. CVID may affect 35% of individuals in USA[13]. Also, 195 CVID and 105 agammaglobulinaemia cases were compared, and the median age of diagnosis was 9.5 and 4 years, respectively[95]. The registration rate of CVID ranges from 0.1 (Russia) to 11 (Netherland) per million population, by country population with a very variable diagnosis at paediatric age (<18) ranging from 0% (Lithuania) to 100% (Russia and Poland, Georgia, Belarus, and Egypt) with a peak of symptoms in the 1st and 3rd decades of life and a diagnosis delay that can reach up to 9 years. This indicates that CVID is a disease that can occur with a similar probability throughout

life and can explain the high rate of bronchiectasis in the CVID, possibly due to diagnostic delay[96, 14,47]. In this report, (n=7, 78%) of CVID and (n=5, 17%) of hypogammaglobulinaemia were diagnosed in adult age.

Severe combined immunodeficiency (SCID) is an emergency medical condition that requires urgent medical interventions like bone marrow transplantation. Most patients with this severe form are diagnosed and die in infancy if no effective treatment is received[94]. No SCID cases were present in this report as the cohort was collected from adult immunology clinics. Only (n=1, 2%) of complement system deficiency is recorded in our cohort. Internationally, the rate is variable and ranges from 0.3 (Japan) to 16.2 (Israel)[28,97]. As SIgAD has an asymptomatic presentation[15], one patient of SIgAD was identified here. The prevalence of PIDD in this cohort is not a representative of the country prevalence as the data were collected from the adult immunology service. This fact explains the non-presence of SCID in our cohort. Moreover, some milder forms as well as patients who were above 14 years and continued to be followed up in paediatric departments were not included.

In this study, the male: female ratio (M: F) was 1.4. The estimated ratio in Qatar is 1.3[98]. In the MENA region, the M:F ranges from 1.1 to 2[28]. Multiple studies conducted in Iran estimated the ratio to be 1.4[27], 4 to 1[97], and 1.7 to 1. In UK the estimated ratio is 1.1[22]. The higher rate in the male gender can be explained by the sex difference in B-cell subsets and immunoglobulin distribution[47]. The global consanguinity rate is 1–9% while it is 20–56% in the MENA region[28]. The parental consanguinity was observed in (n=7, 14.6%) in this study, and although it is lower than the average in the MENA region, it continues to be higher than the global average.

The complications reported in PIDD varies and can involve respiratory, musculoskeletal, cutaneous and gastroenterology systems[97]. The antibody deficiency is considered a very rare cause of bronchiectasis with estimation of 0.7%-2.4% (adult) and 2%–10% (paediatric) when studied done in respiratory services[99]. However, higher rate is observed when analysing data about bronchiectasis among PIDD patients. The rate of bronchiectasis was reported to be 42.2% (of the 192 CVID) and 32.2% (of the 102 agammaglobulinaemia)[95]. In this report, recurrent infections were the main presentation as well as the main complications and the rate of bronchiectasis was 100% in CVID and 50% in hypogammaglobulinaemia.

Among the clinical parameters that been evaluated in relation to the two most common complications; bronchiectasis and allergy in B-cell PIDD, it was found that the age of presentation to the health service was the most reliable indicator for both complications. The presentation in paediatric age group (<14 years old) was associated with higher rate of bronchiectasis (*P*=0.002) while allergy complication was higher in those presented in Adult age (*P*=0.023). This may infer that the severe phenotypes presenting in childhood with recurrent and more severe infections lead to earlier physician suspicion and a diagnosis of the underlying immunodeficiency. The presentation with symptoms suggestive of asthma and other allergic disorders that are common childhood disorders may cause physicians to skip the investigation of the possibility of immunodeficiency until adult age. Male gender was associated with a higher bronchiectasis rate than female (*P*=0.003).

The blood chemical biomarkers were evaluated in B-cell PIDD in relation to those complications. Most of the vitamins examined did not show any association. A high level of vitamin D deficiency was found in the Ataxia-telangiectasia group 6/14 (42.8%) and CVID group 3/17 (17.6%)[70]. The same low level was observed in this study and vitamin D was low in 91.7% of B-cell PIDD. However, it was not associated with an increased rate of any of the complications. Human studies to evaluate dietary effects on immune systems are very limited. Also, most of these studies depend on the blood testing[38,69].

C-reactive protein (CRP) is a very strong biomarker. Commonly it raised during inflammation (e.g. rheumatoid arthritis), cardiovascular diseases, and infections by up to 1000-fold during tissue damage or disease progression; it then falls after the reaction subsides within 18-20 hours[100]. In our data, CRP and initial IgG level were statistically significant in both complication groups (*P*<.05). Moreover, the mean and median White Blood Cell (WBC) counts were statistically higher in patients without complications (*P*=0.033); this may indicate a lower infection rate in this group compared to the bronchiectasis complication group, possibly due to good immunological cell response showed by an increase in WBC number. An interesting observation of the results is that the elevated CRP was associated with bronchiectasis while it was associated with no allergy complications. The opposite was observed for the initial IgG, which was lower in the bronchiectasis complications and no allergy complications group.

The questionnaires were conducted to help with the selection of patients for the interventional stage of the study. More than 48 types of bias can be faced when looking at the questionnaires

and their analysis[101]; the most important in our data are recall bias and the response fatigue due to long questionnaires. However, potential candidates were identified, and 10 patients were selected successfully. The analysis that was done showed equal gender distribution and that most of the patients adapted a healthy life style with the sufficient consumption of antioxidants: fruit and vegetables (mean ± SD) accounted for 4.12 ± 4.41 and 6.16 ± 3.26 of daily servings, respectively. Dominating symptoms were cough and fatigue, sluggishness and swing of the mood. However, most patients were leading an almost normal life (despite the chronicity of PIDD) similar to that of the healthy population based on the quality of life and mental health questionnaires. The Spearman's correlation between diet compositions to age and BMI indicate distorted reverse relation between sweet intake and weight. There is no clear explanation for this identified phenomenon and it is possible that obesity was related to eating other food like snakes, cereals etc.; however, the relation was not shown to be statistically significant. The Pearson's correlation analysis between age, gender, and BMI and the different symptoms scores showed and a positive correlation with difficulty in making decision, and frequent illnesses and a negative correlation for faintness, hair loss, perception of excessive weight, and frequent urination in relation to age and BMI. No symptoms were significantly correlated to gender. We believe this is a logical correlation and can be reported in any chronic disease, rather than just being specific to PIDD.

The findings of this study have to be seen in light of some limitations. Despite the study cohort representing all patients registered over the last 10 years in adult immunology service in Qatar, the small sample size of patients (n=48) is considered a limitation to the generalisation of results. Also, the population in this study is not reflective of the total general population of PIDD as it is retrieved from adult immunology clinics in Qatar. In general, there are limited research studies and significant lack of awareness about adult PIDD. PIDD is a group of rare disorders, particularly in the adult population. It may be worth collecting more data in the future to examine PIDD in the adult population by doing different types of research, like conducting an epidemiological survey to identify the real prevalence in Qatar. Another future direction could be multicentre studies to evaluate the burden of PIDD disease in the adult populations. Another limitation to the study was using the questionnaires. It is well known that multiple biases can be faced when looking at the questionnaires, including cultural bias and other personal issues[101]. The dietary questionnaires in particular carry multiple biases and cofounding factors, including the use of closed-ended questions and recall bias that may affect the accuracy. Also, it takes a long time for patients, who are suffering from chronicity and multiple disease-related complications of PIDD because of the

long list of food items. However, it may be time-saving somehow compared to daily records and simple to use and to gather usual dietary intake and to have estimate for average dietary habits over long period of time.

3.7. Summary

Patients with recurrent episodes of infections should receive an evaluation of the immunologic system early to fill the gap in late diagnosis and consequently to improve the treatment of PIDD patients and reduce the infection rate and complications. Familiarising the paediatric and medical physicians about primary immunodeficiency disorders is challenging across the globe because of the disease rarity and the focus of most health academy and community on other more prevalent morbid diseases. However, it is highly mandated to incorporate the knowledge of PIDD into the practice of general practitioners and primary and first line care providers.

Further specific and pertinent analysis of the interventional stage to investigate the possibility of diet change as an immunomodulator and a potential area to invest in during the care of patients with primary immunodeficiency patients particularly humeral, is presented in Chapter 4.

CHAPTER 4

RESULTS. MiRNA AND MICROBIOME ANALYSIS

4.1. Introduction

This is the second of the two results chapters. This chapter provides an overview of data gathered in relation to the gut microbiome and serum exosome microRNA laboratory work analyses of ten patients with predominantly antibody deficiency that were recruited into the study. The results before and after interventions with micronutrient supplementations and intravenous immunoglobulin administration were compared.

Microbiome and microRNA received an interest as important controlling points in inflammation and are targeted alone or combined in multiple studies[102-104] to help with understating the mechanisms of immunomodulation, inflammatory responses and autoimmunity[103-104]. The diet-microbe-metabolite-host interactions are studies to unravel targets for potentially modulate inflammatory responses and autoimmunity[105-107].

microRNA (miRNA or miR) are shown to play important roles at different stages of the development of the immune system for both innate and adaptive immunity[103] (Figures 17 & 18). A recent review found that microRNAs (miRNAs) are essential for B-cell homeostasis, and this area can be a promising for identifying biomarkers and therapeutic options to cure B-cell-related immune disorders[108]. Only a few studies have tried to determine the role of microRNA and microbiome in PAD and as potential future therapeutic options[71,54,109].

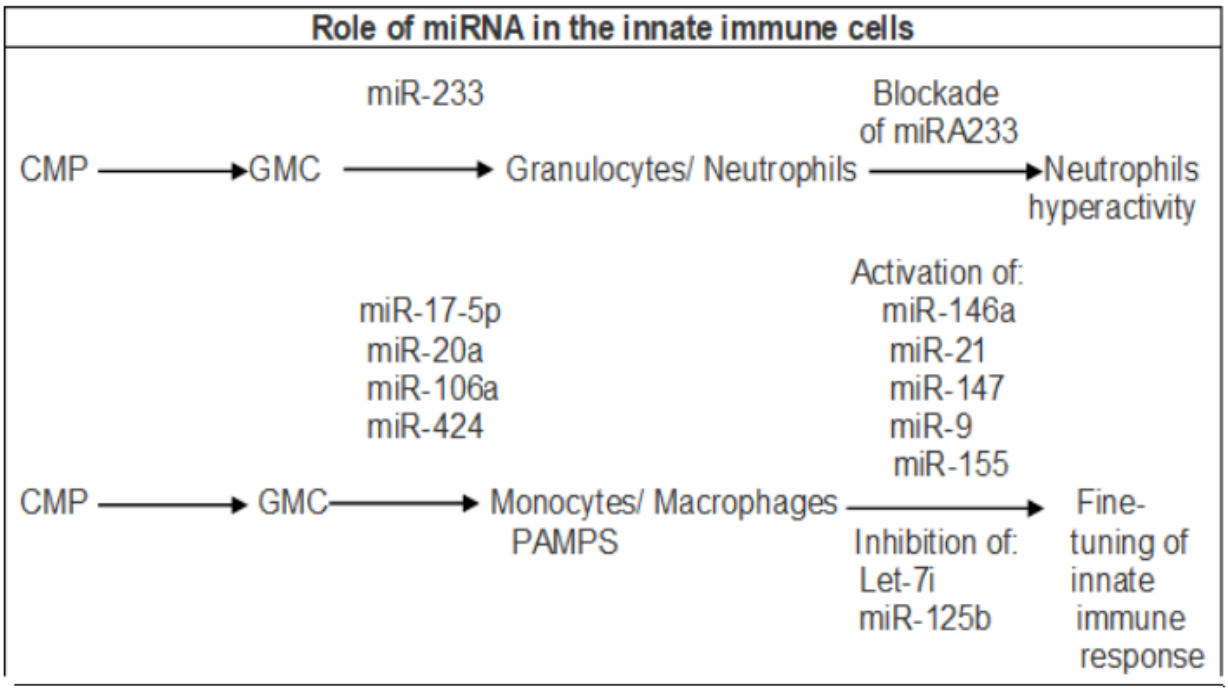


Figure 17: miRNAs involved in the development and function of the innate immune system. (Note; a*dapted from Dai et al. 2011*[103]).

CMP: common myeloid progenitor, GMP: granulocyte-monocyte progenitor, PAMPs: pathogen-associated molecular patterns.

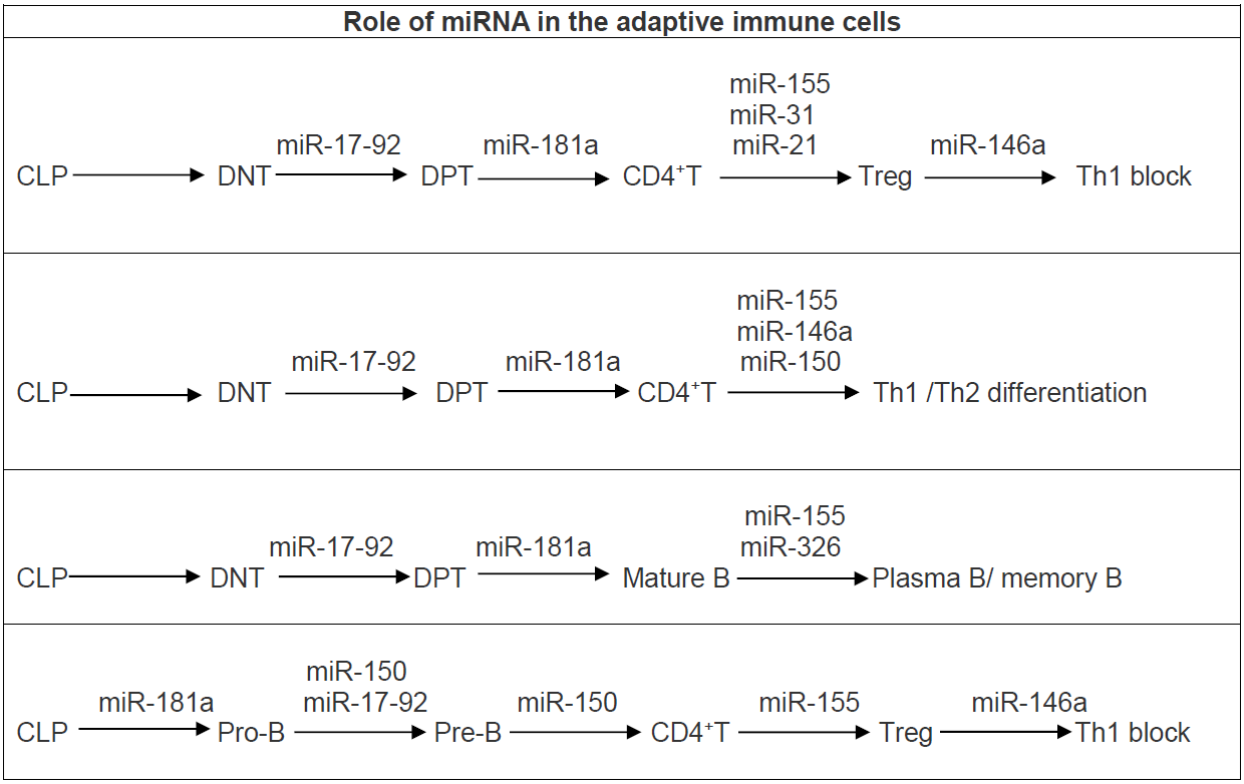


Figure 18: miRNAs involved in the development and differentiation of adaptive immune system.

(*Note: adapted from Dai et al. 2011*[103]).

CLP: common lymphoid progenitor, $CD4^-$ $CD8^-$: double negative (DN), $CD4^+CD8^+$: double positive (DP), Treg: regulatory T cell.

4.2. Aim of the study

1. To investigate the effect of supplementation with common micronutrients (multivitamins A, B, E and D), alone or combined with IGIV, on the serum exosome microRNA and gut microbiome.

4.3. Methods

Ten patients were selected based on the questionnaire analysis (see Chapter 3 section 2.1.4 - 2.1.5 and 2.2.2). The selected patients fulfilled the inclusion clinical criteria for Predominantly Antibody Deficiency (CVID and SAD). The internationally recommended dose of multivitamin supplements: Vit-A, -E, -B & -D for an eight-week duration (intervention period). Serum exosome miRNA and stool microbiome were extracted and analysed. The blood and faecal samples were collected at three intervals; before, by the end of 8 weeks, and 6 weeks after stopping vitamin supplementation.

4.4. Results: Demographic Characteristics of the 10 patients recruited into the interventional stage

Written informed consent was obtained from all participants in the study. The study was approved by local Ethical Committee of the Hamad Medical Corporation, Doha, Qatar (MRC-01-17-029). All clinical investigations were conducted according to the principles expressed in the 1964 Helsinki declaration and its recent amendments. Demographic characteristics of the ten patients recruited into the study is presented in the Tables 18 and 19.

#	Age	Diagnosis Maturation	BMI	smoking	Sex	Ethnicity
P1	24	adult	16.66	no	female	Arab
P2	14	adult	17.7	no	male	Arab
P3	23	adult	20.83	no	female	Arab
P4	21	adult	20.04	no	female	Arab
P5	36	adult	28.26	no	female	Arab
P6	34	adult	26.87	no	female	Arab
P7	25	paediatric	17.08	no	female	Arab
P8	43	adult	42.04	no	male	Arab
P9	28	paediatric	18.9	no	female	Arab
P10	26	adult	21.61	no	female	Arab

Table 18: Demographic characteristics of the study population.

Description of the 10 patients that were recruited into the interventional study. Patient sequence number was the same sequence number that been entered into array analysis of the microbiome and microRNA that will be presented in the next chapter.

#	Disease subclass	Complications	Hours before sample reach freezer	Courses of antibiotics in the last year before recruitment	IGIV use (3 months vs. long-time
P1	SAD	recurrent infections	2	on prophylactic antibiotics	short term
P2	SAD	recurrent infections	2		short term
P3	CVID	recurrent infections, arthritis	4	on prophylactic antibiotics	short term
P4	SAD	recurrent infections	4	2	short term
P5	CVID	recurrent infections	2	5	short term
P6	CVID	asthma and urticaria, recurrent infections	3.5	3	short term
P7	CVID	recurrent infections	4	3	long term
P8	CVID	lymphoma, recurrent infections	4	on prophylactic antibiotics	long term
P9	CVID	recurrent infections	3.5	4	long term
P10	CVID	recurrent infections, thrombocytopenia	2	2	long term

Table 19: Clinical parameters of the study population.

Recurrent infections were presented in all patients. Four out of the ten patients were using Intravenous Immunoglobulin (IGIV) medication since childhood. The remaining 6 patients started to receive IGIV three months prior to collecting the second blood samples. The first blood samples were collected from those six patients before starting IGIV treatment. Patient sequence number was the same as that which had been entered into array analysis of the microbiome and microRNA that will be presented in the next sections.

4.5. Results: Microbiome

4.5.1. Introduction

Microbiome analysis was conducted using Axiom™ MiDAS.

High-resolution genomic identification of archaea, bacteria, fungi, protozoa, and viruses can be detected in complex microbial samples.

Adapted from ThermoFisher Scientific website; accessed 1/1/2019 (https://www.thermofisher.com/qa/en/home/life-science/microarray-analysis/microarray-analysis-instruments-software-services/microarray-analysis-software/axiom-microbial-detection-analysis-software.html)

Microbiome data were analysed and expressed using alpha and beta diversity. The definition of each one and the results were given below in **section 4.5.5** (**alpha diversity)** and **section 4.5.6 (beta diversity)**.

4.5.2. Microbiome analysis plan

Figure 25 and Table 20 summarised the analysis plan sample condition for the 44 microbiome faecal samples (for 10 patients).

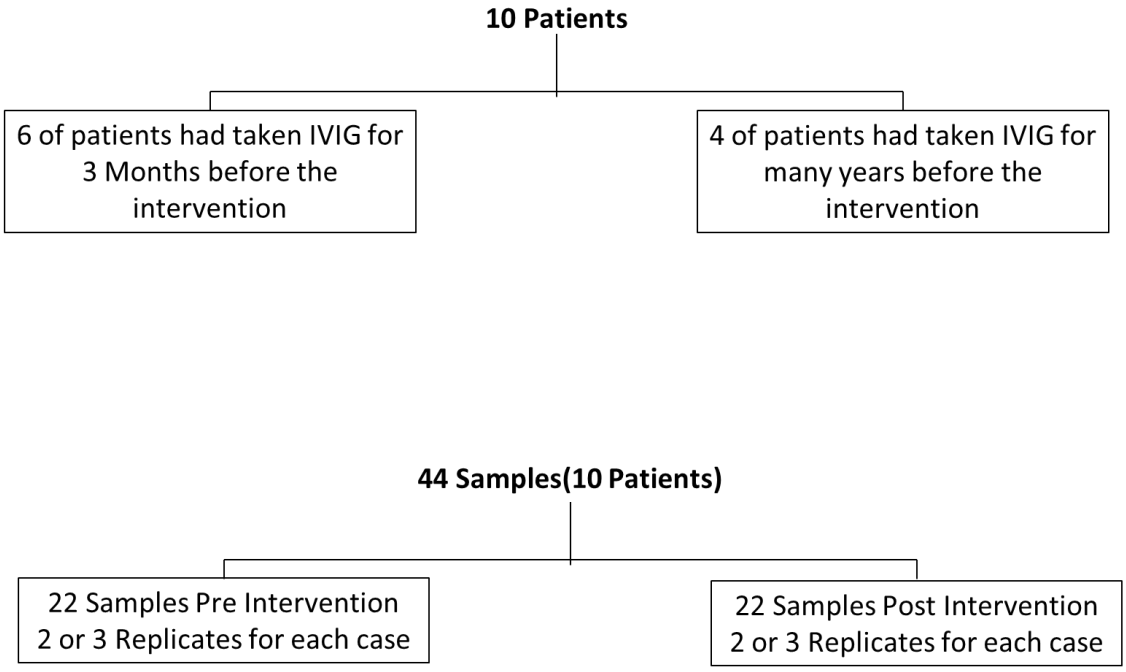


Figure 19: Analytical plan of the microbiome faecal samples.

Analysis of the microbiome (n= 44) faecal samples, representing 10 patients, for alpha and beta diversity analysis. The two positive and two negative controls used during running the array plate have been removed from this analysis.

Patient number	condition		Duplicate		Triplicate		Total
	Post IgG-infusion/ pre-vitamins	Post combined IgG and vitamins	Post IgG-infusion/ pre-vitamins	Post combined IgG and vitamins	Post IgG-infusion/ pre-vitamins	Post combined IgG and vitamins	
P1	P1a_PreVit	P1a_PostVit	P1b_PreVit	P1b_PostVit			4
P2	P2a_PreVit	P2a_PostVit	P2b_PreVit	P2b_PostVit			4
P3	P3a_PreVit	P3a_PostVit	P3b_PreVit	P3b_PostVit			4
P4	P4a_PreVit	P4a_PostVit	P4b_PreVit	P4b_PostVit			4
P5	P5a_PreVit	P5a_PostVit	P5b_PreVit	P5b_PostVit			4
P6	P6a_PreVit	P6a_PostVit	P5b_PreVit	P5b_PostVit			4
P7	P7a_PreVit	P7a_PostVit	P7b_PreVit	P7b_PostVit	P7c-PreVit	P7c_PostVit	6
P8	P8a_PreVit	P8a_PostVit	P8b_PreVit	P8b_PostVit			4
P9	P9a_PreVit	P9a_PostVit	P9b_PreVit	P9b_PostVit			4
P10	P10a_PreVit	P10a_PostVit	P10b_PreVit	P10b_PostVit	P10c_PreVit	P10c_PostVit	6
DNA -C	2						2
NTC	2						2
Total	10	10	7	7	2		48

Table 20: Microbiome conditions on the microarray

All patients faecal sample were done in duplicate except P7 and P10 that were done in triplicate on the plate array. 22 samples and two (one negative and one DNA positive) controls were run in each of the 24-microarray plates used in this study.

4.5.3. Microbiome Quality assessment

4.5.3.1. Assessing the quality of genomic DNA using 1% Agarose E-gels before Genetitan application

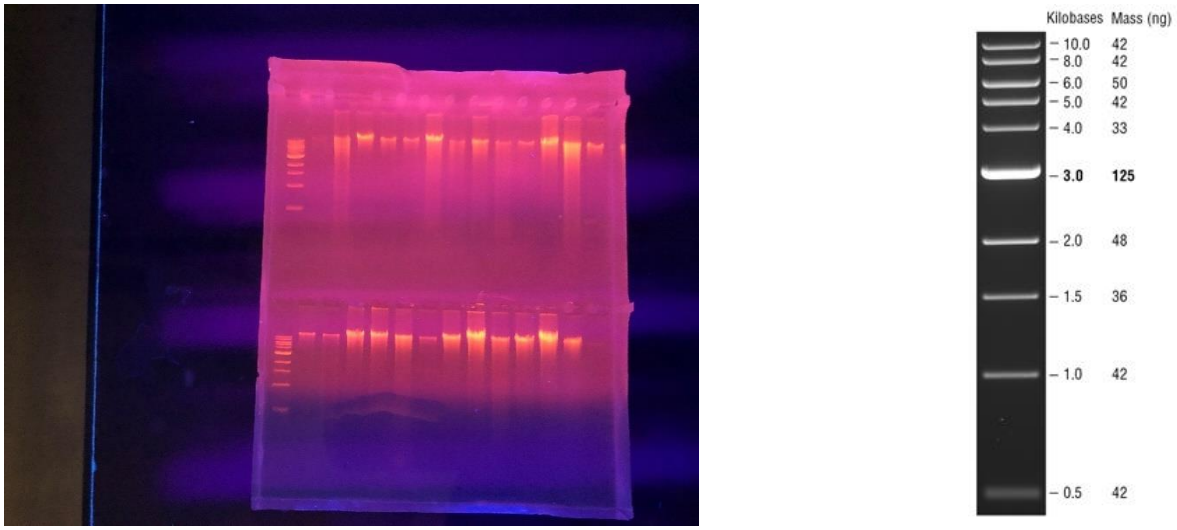


Figure 20: The quality assessment of the integrity of genomic DNA for Genetitan application.

The figure showed good and intact DNA. DNA must not be degraded. The approximate average size of gDNA was assessed on a 1% agarose gel using an appropriate size standard control. Approximately 90% of the DNA must be greater than 10 Kb in size. Refer to Figure 10C in chapter 2 (section 2.2.2.4.2.1.) for further details about methodology.

4.5.3.2. Microbiome DNA assessment using the Nanodrop

Faecal microbiome DNA was assessed using Nanodrop reading after extraction, cleaning up with ethanol and heating att 37ºC. It showed good values that were acceptable based on the manufacturer's requirements. Refer to chapter 2 (section 2.2.2.4.) for the utilisation of Nanodrop methodology and to **appendix VIII** for specific samples value of the Nanodrop reading.

4.5.3.3. Quality assessment during array preparation for fragmentation (QC checks using 3% agarose gel)

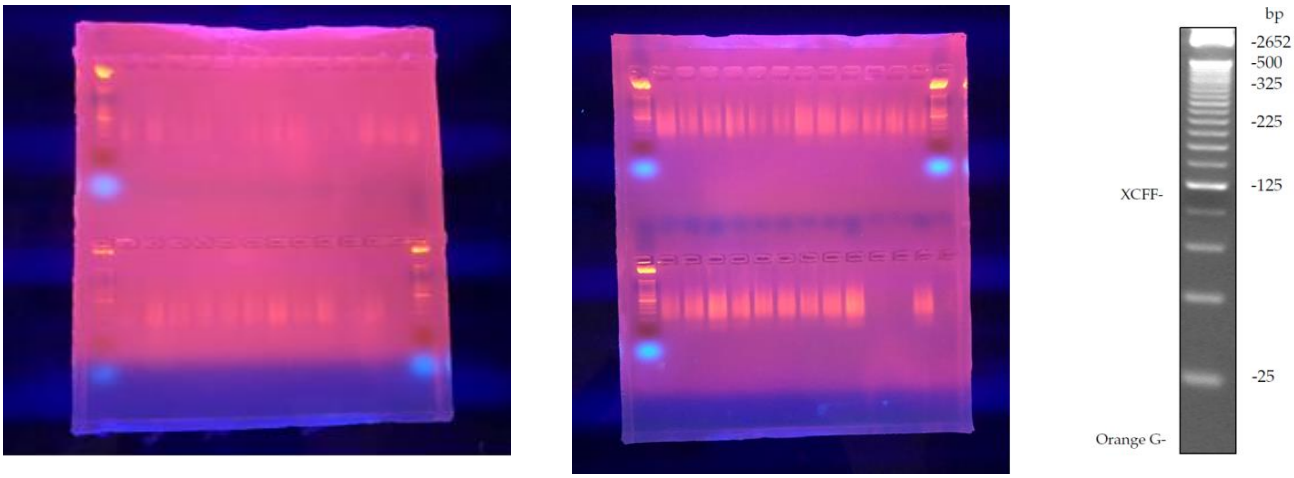


Plate 1 **Plate 2**

Figure 21: The quality assessment of the fragmentation QC checks.

The quality of fragmentation was assessed using 3% agarose gel. During microarray preparation, stage one of DNA amplification was followed by stage two of fragmentation, precipitation, and stage three of centrifuge and drying, resuspension and hybridisation preparation. Before proceeding to Stage 4 of denaturation and hybridisation, a quantitation and fragmentation quality control check was carried at this stage using 3-4% agarose E-gel. Refer to Figure 10d of chapter 2 (section 2.2.2.4.2.2.) for an example of the QC check methodology.

4.5.3.4. Quantification of samples post-amplification and defragmentation and before the denaturation and hybridisation steps

The Nanodrop was used to perform an assessment of the amplification and defragmentation step that should be done before proceeding to next step of denaturation and hybridisation. It is expected to achieve 120-fold mass dilution)

It was found that this step was successful with all samples that reached 120-fold mass dilutions and allowed to proceed for next step in the microbiome protocol. Refer to **appendix IX** for detailed results of the Nanodrop quantification

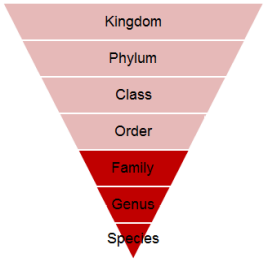
4.5.4. The Detected Targets

4.5.4.1. Total Targets detected

Sample Name	Number of Targets Detected						Number of Species Detected						Number of Genera Detected						Number of Families Detected					
	Pre			Post			Pre			Post			Pre			Post			Pre			Post		
	S	D	T	S	D	T	S	D	T	S	D	T	S	D	T	S	D	T	S	D	T	S	D	T
P1	53	58		53	57		48	53		46	51		34	36		32	35		13	14		13	16	
P2	31	33		53	48		28	30		46	44		18	19		29	29		10	10		14	13	
P3	51	52		57	52		45	43		47	43		30	30		33	29		19	20		19	16	
P4	49	51		32	35		46	48		32	33		32	32		25	25		18	18		12	13	
P5	58	53		40	39		44	40		32	30		23	23		19	19		15	15		13	14	
P6	62	57		71	72		49	43		56	56		30	26		32	35		21	18		21	20	
P7	32	43	40	31	36	33	26	36	33	29	33	32	22	26	27	20	21	20	13	20	19	13	13	13
P8	39	49		75	50		36	43		61	44		22	22		37	28		17	17		24	20	
P9	48	44		49	57		42	39		44	52		30	28		36	41		19	18		21	21	
P10	44	39	47	62	60	58	39	36	41	56	56	53	29	25	27	38	37	35	17	16	15	18	19	18

Table 21: Summary of the number of targets detected.

The Axiom™ Microbial Detection Analysis Software (MiDAS) was used. The list summarised the three most discrete levels of taxonomic resolution.The table allows an initial analysis of each sample composition.



In this summary table, reference to terminology is listed below:

Sample Name: Name of the sample CEL file. **Array Type**: Microarray type. **Number of Targets Detected**: Number of targets in the algorithm model. **Number of Species Detected**: Number of species in the algorithm model. **Number of Genera Detected**: Number of genera in the algorithm model. **Number of Families Detected**: Number of families in the algorithm model. **S**: Sample. **D**: Replicate (duplicate) for the same sample. **T**: Replicate (triplicate) for the same sample.

The number of families shown here only represent targets with known family assignments from the Axiom™ MiDAS database.

(Analysis is based on Analysis of Axiom™ MiDAS results (chapter 4), Axiom™ Microbiome Solution. USER GUIDE. Catalogue Numbers 902910, 902904, and 902903. Publication Number 703408. Revision 2.

Adapted from thermofisher scientific website, accessed on 19/06/2019 (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/703408_Axiom-Microbiome-UG.pdf)

4.5.4.2. Families identified and those which disappeared after intervention

Further analysis of the targets detected (by family's name) that were observed to be changed by the intervention step is summarized below tables 22 & 23.

4.5.4.2.1. Families show-up after intervention

Family Detected	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Acidaminococcaceae		√								
Bifidobacteriaceae		√								√
Coriobacteriaceae						√				√
Desulfovibrionaceae								√		
Enterobacteriaceae		√								
Erysipelotrichaceae			√			√		√		√
Eubacteriaceae								√		
Lachnospiraceae								√		
Leuconostocaceae						√				
Micrococcaceae										√
Oxalobacteraceae									√	
Porphyromonadaceae								√		
Rikenellaceae								√		
Streptococcaceae		√								
Veillonellaceae				√						
Myoviridae								√		
Unassigned bacteria								√		
Unassigned virus								√		

Table 22: Microbial families detected (show-up) only in the post-intervention samples.
These were not present before intervention (micronutrients supplementation in pre-intervention samples (both duplicates).

4.5.4.2.2. Families not present after intervention

Family Detected	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Bifidobacteriaceae					√					
Clostridiaceae			√	√			√			
Coriobacteriaceae			√	√						
Desulfovibrionaceae										√
Enterobacteriaceae				√						
Eubacteriaceae							√			
Leptotrichiaceae								√		
Myoviridae				√						
Oscillospiraceae							√			
Oxalobacteraceae				√						
Podoviridae							√			
Prevotellaceae							√			√
Rikenellaceae							√			
Streptococcaceae					√					
veillonellaceae		√								
Verrucomicrobiaceae				√		√				

Table 23: Microbial families not apparent post intervention.

These were present before Intervention (micronutrients supplementation) in pre-intervention samples (both duplicates).

Results Summary for Tables 22 & 23

The tables summarise targets (families) identified in each patient. Family targets were found to be different in the post-intervention compared to pre-intervention samples.

The erysipelotrichaceae family represented the most abundant microorganism post intervention out of those that were absent before and detected in the faecal samples after intervention (in 4 patients). Patient 8 developed more than the other patients (9 new organisms) that were not observed before the intervention. In contrast, the Clostridiaceae family represented the most abundant microorganisms that were present before and after the samples are feft after intervention (in 30 samples). Patient 4 & 7 lost more than other patients (6 new organisms) that were detected before the intervention.

4.5.4.3. Microbe Frequencies

Figures 28 and 29 summarise the commonest 35 microbes' frequencies based on the species and family microorganism's taxonomy.

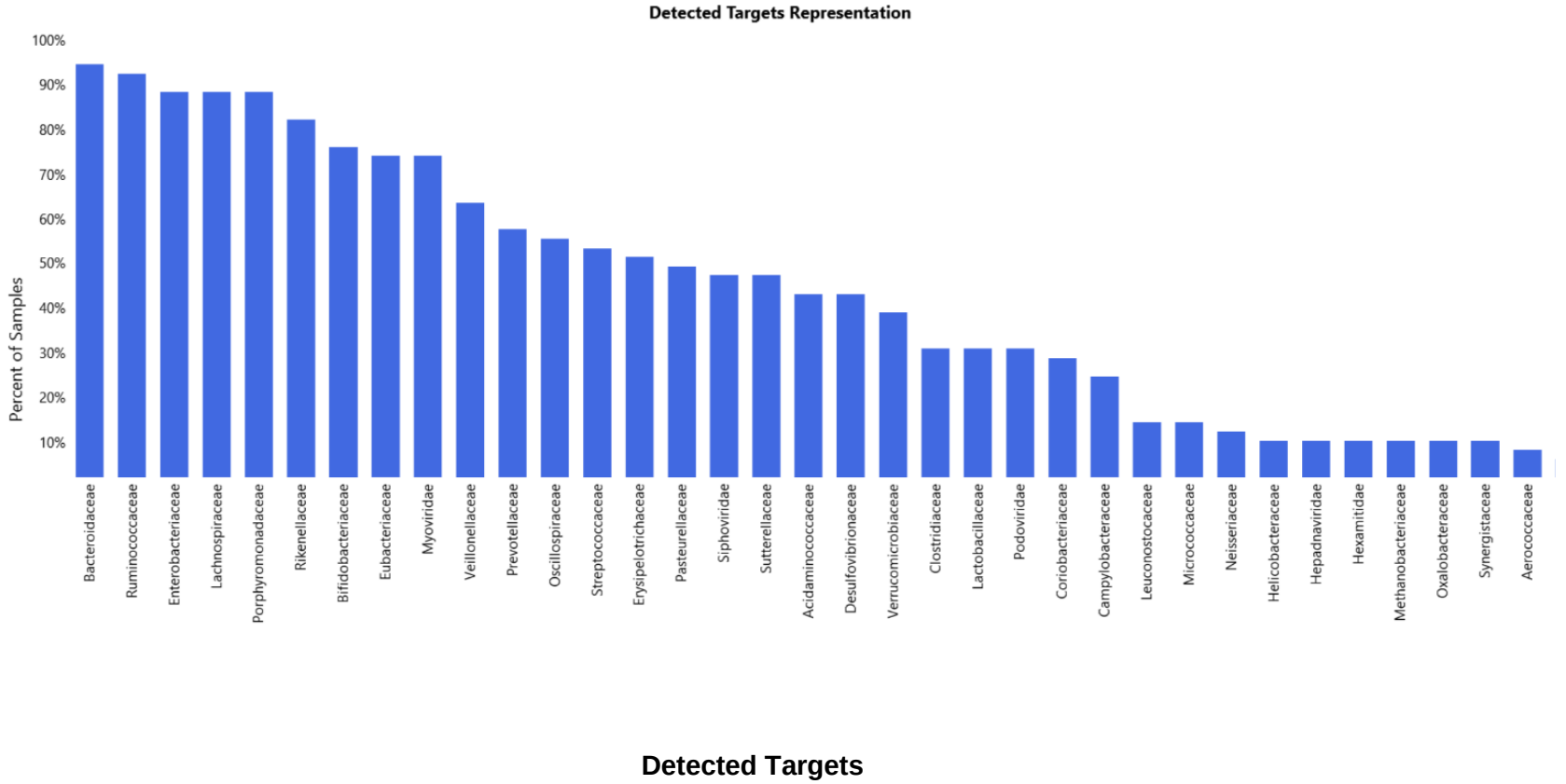


Figure 22: Frequencies based on detected targets microbial species.

The first 35 frequencies based on microbial species n=46 samples (10 patients).

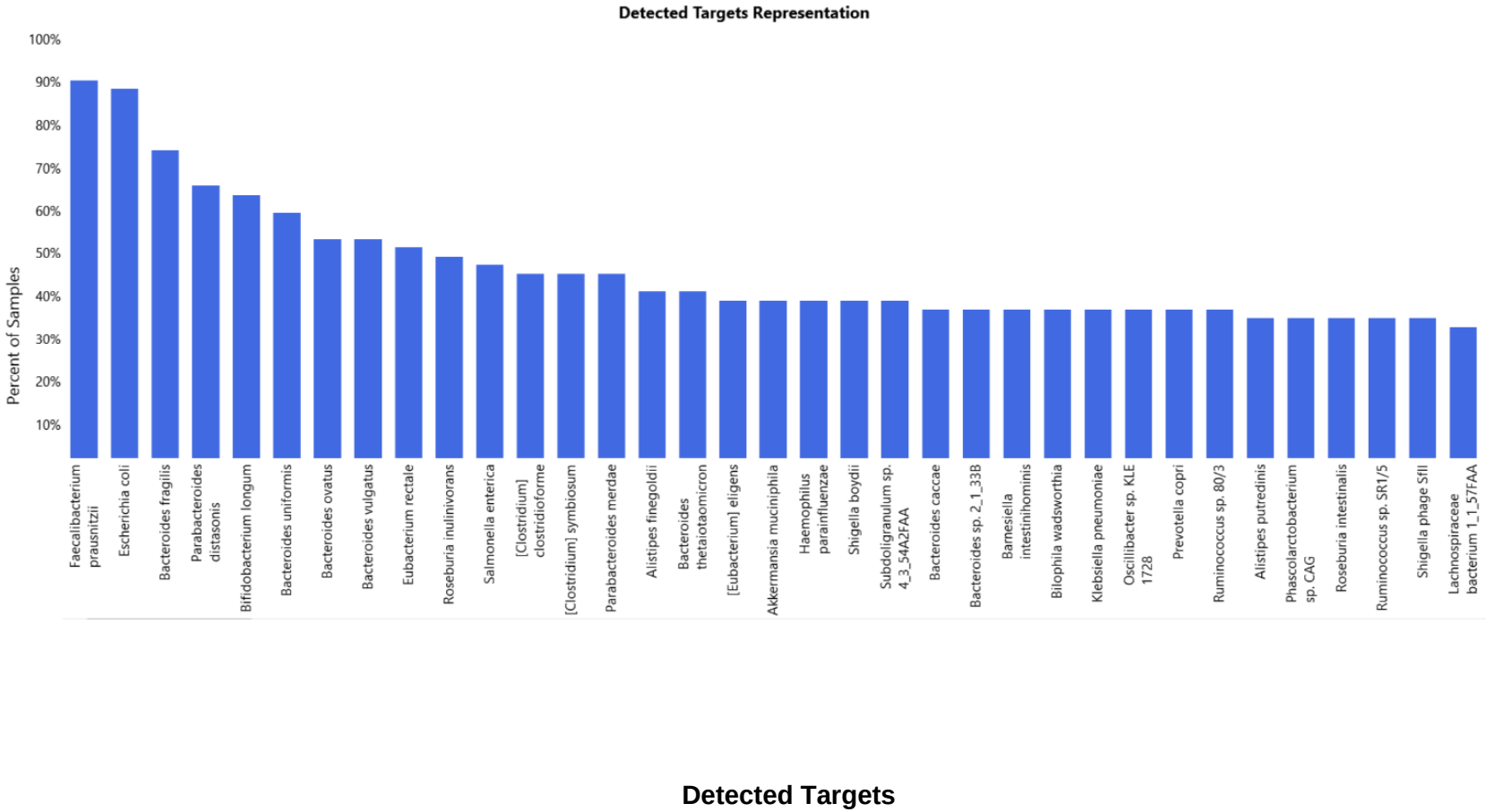


Figure 23: Frequencies based on detected targets of microbial families.

The first 35 frequencies were based on microbial families, n=46 samples (10 patients).

4.5.5. Alpha Diversity

For the alpha diversity, the observed alpha diversity was used (https://drive5.com/usearch/manual/alpha_diversity.html)[110] which measures how many targets (bacteria or viruses presented in this sample)

The terms alpha and beta diversity were introduced by R.H. Whittaker in 1960.

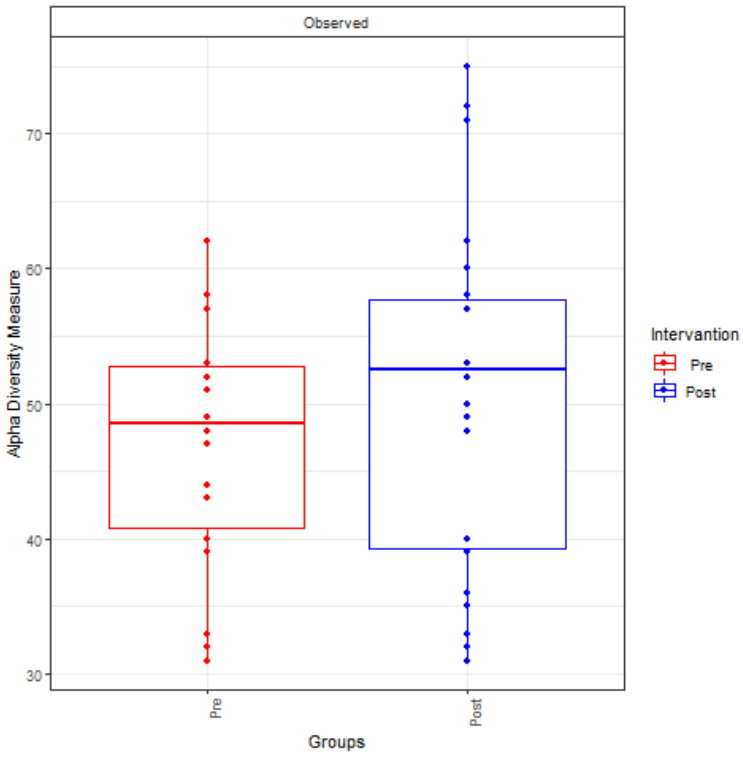
Alpha diversity is the diversity in a single ecosystem or sample. The simplest measure is the species richness, the number of species (or OTUs) observed in the sample. It is the answer to the question how many different species could be detected in a microbial ecosystem? So, species richness is the number of different species in a sample. Practically, we count the number of distinguishable taxa (OTU's) in each sample.

Other metrics consider the abundances (frequencies) of the OTUs, i.e. the proportion of a species in a community. For example, to give lower weight to lower-abundance OTUs. Axiome microbiome is a sequencing array which is not able to give abundances. Also, evenness that is how well a species represented is another index to measure alpha diversity[110]. **Appendix X give details about commonly used diversity metrics**

For alpha diversity: We utilized R Package (microbiomesick) for microbiome:

https://www.bioconductor.org/packages/release/bioc/html/phyloseq.html

4.5.5.1. Alpha diversity among patients before and after intervention with micronutrients



n=10

44 Samples

Figure 24: Alpha diversity in patients with PAD after vitamin supplementation.

Analysis was done for all samples, pre- vs. post-intervention with vitamins supplements. The results were presented in median, quartiles, and range. The comparison was made by using *t*-test. Any box plot shows significant *p*-value, is marked as *** <0.001, ** <0.01, * <0.05.

Results Summary: There was no significant difference in the observed diversity. However, the post samples were more diverse than the pre sample. More diverse means more targets were detected.

4.5.5.2. Alpha diversity comparing patients received short term (IGIV for the last three months) vs. those on long term (IGIV for many years)

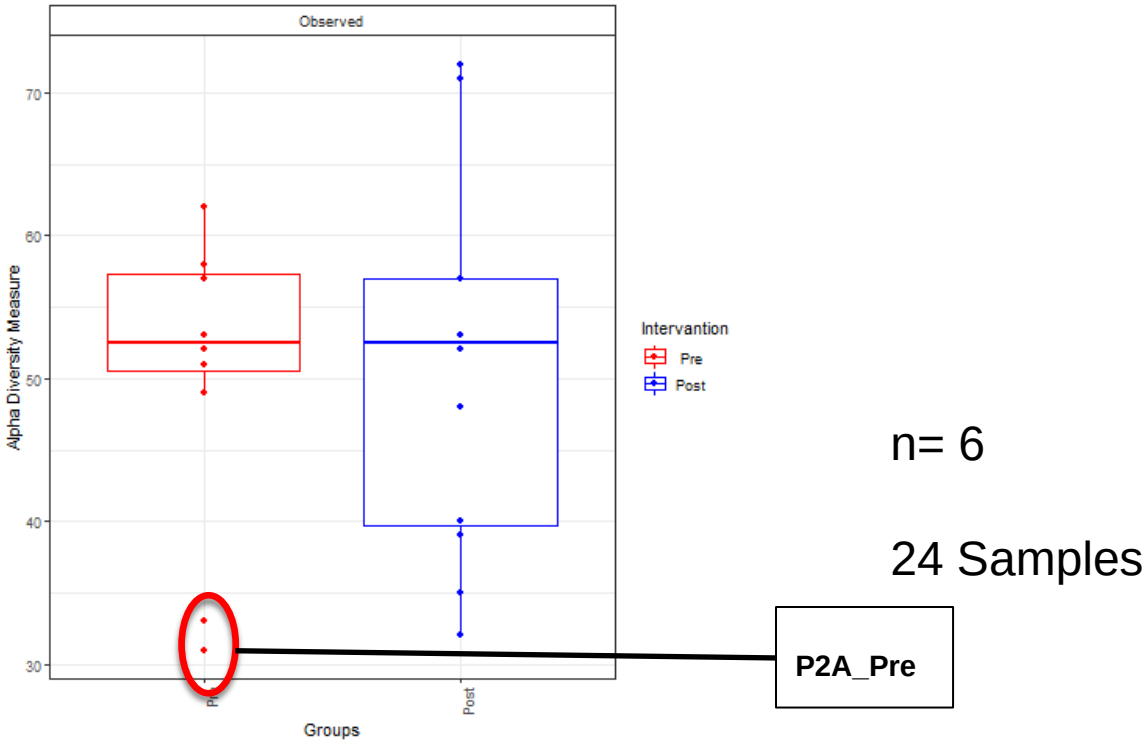
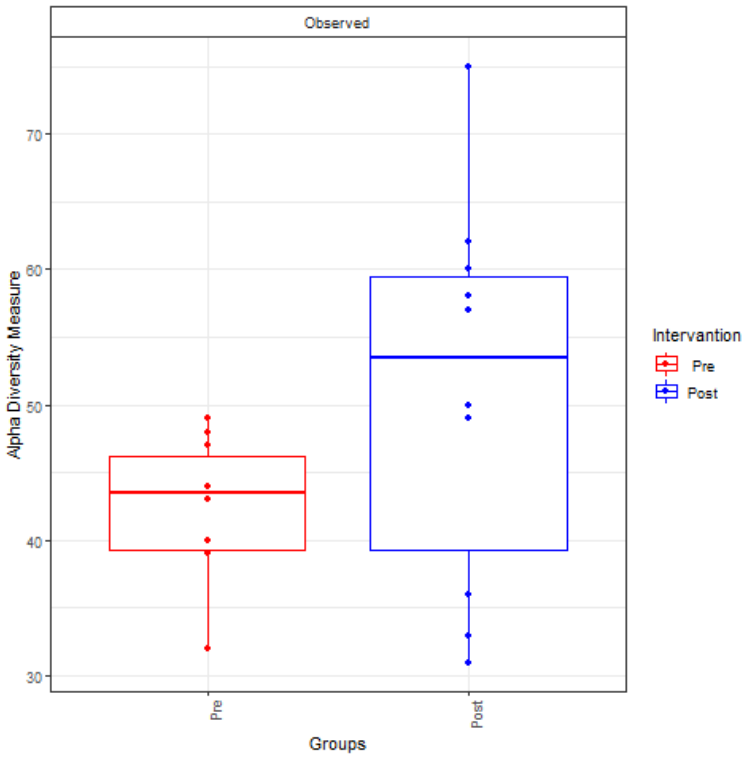


Figure 25: Alpha diversity in patients with PAD after short-term IGIV.

Analysis was done to compare pre- vs. post-intervention with vitamins supplements in the subgroup of patients that were in short term IGIV. The results were presented in median quartiles and range. The comparison was made by using *t*-test. Any box plot that showed significant *p*-value, is marked as *** <0.001, ** <0.01, * <.05.

Results Summary: There was no significant difference in the observed diversity. The Post samples diverse almost equal to Pre samples diverse. In reviewing the medical and blood tests as well as procedures of stool collection for the outliers, no explanation could be identified.



n=4

20 Samples

Figure 26: Alpha Diversity in patients with PAD after long-term IGIV.

Analysis was done to compare pre- vs. post-intervention with vitamins supplements) in the subgroup of patients that were in long-term IGIV. The results were presented in median quartiles and range. The comparison was made by using *t*-test. Any box plot shows significant *p*-value, is marked as *** <.001, ** <.01, * <.05.

Results Summary: There is no significant difference in the observed diversity. The Post samples were more diverse than the Pre samples. More diverse meaning more targets detected

4.5.6. Beta Diversity

For all the Beta diversity the (http://rfunctions.blogspot.com/2016/08/measuring-and-comparing-beta-diversity.html)[111] was used for definition.

Beta diversity cab be defined by asking how different is the microbial composition in one environment compared to another. So, it is the diversity in microbial community between different environments (difference in taxonomic abundance profiles from different samples). Two metrics can be utilized to measure the beta diversity: Sorensen Index (also known as Bray-Curtis) and Jaccard Index[111].

Sorensen's Index is a measure that is very similar to the Jaccard measure. Sorensen and Jaccard coefficients are very closely correlated[111].

Jaccard distance is based on the presence or absence of species (does not include abundance information). It can be defined as the different in microbial composition between two samples where 0 means both samples share exact the same species and 1 means both samples have no species in common[111].

For beta diversity: We utilised the R Package (betapart) for the microbiome:

https://cran.r-project.org/web/packages/betapart/index.html

https://cran.r-project.org/web/packages/betapart/betapart.pdf

4.5.6.1. Beta diversity among patients with PIDD before & after intervention with micronutrients

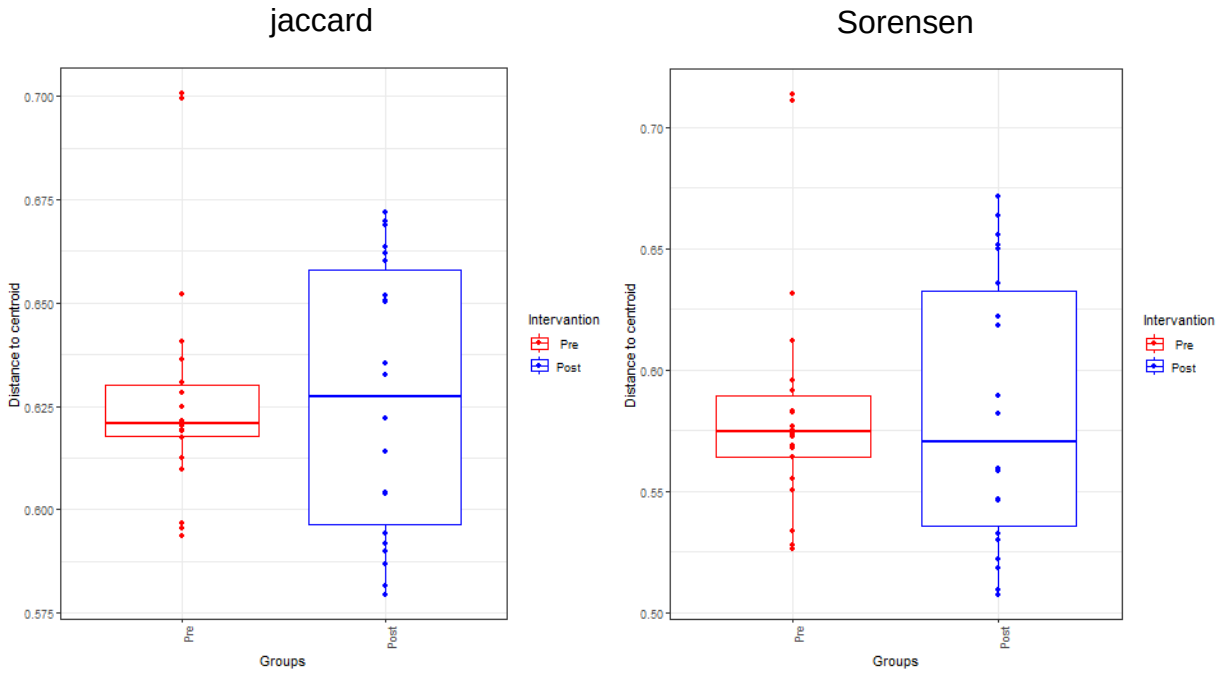
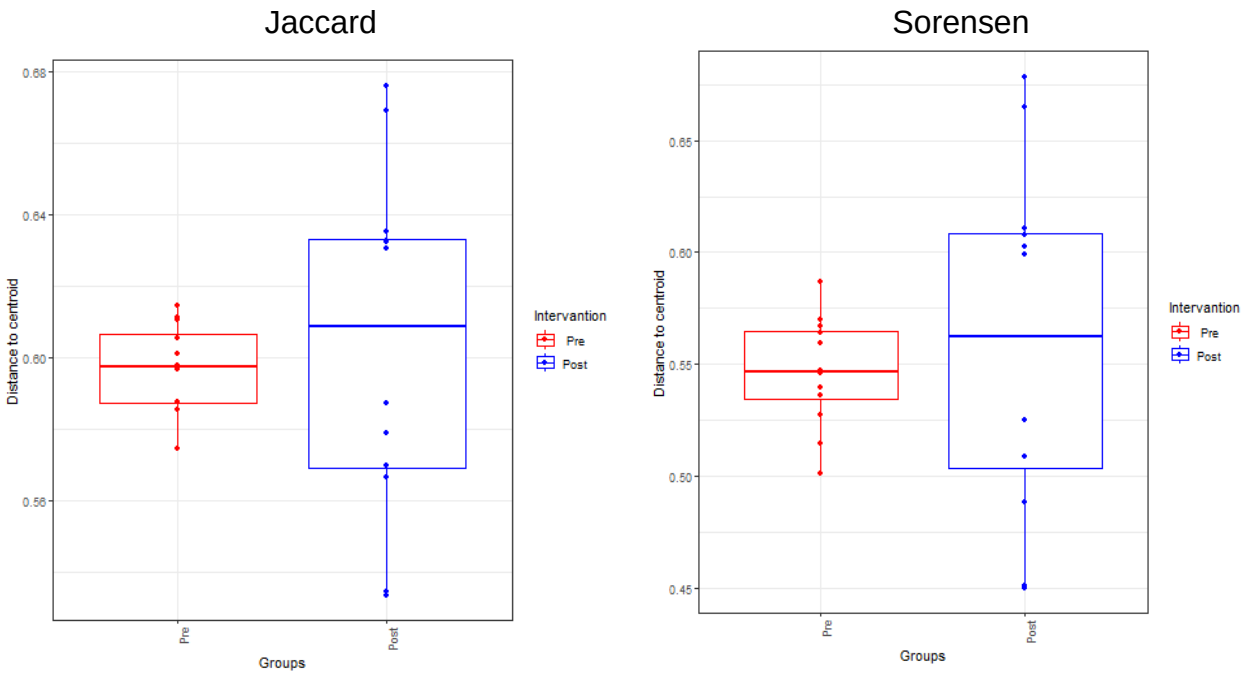


Figure 27: Beta Diversity in patients with PAD after vitamin supplementation.

Analysis was done of all samples, pre- vs. post-intervention with vitamin supplements. The results were presented in median, quartiles, and range. The comparison was made by using *t*-test. Any box plot shows significant *p*-value, is marked as *** <0.001, ** <0.01, * <0.05.

Results Summary: There is no significant difference in the observed diversity. However, the Post samples were more diverse than the pre samples by both Jaccard and Sorensen. More diverse means more targets detected.

4.5.6.2. Beta diversity comparing patients received short term (IGIV for the last three months) vs. those on long term (IGIV for many years)

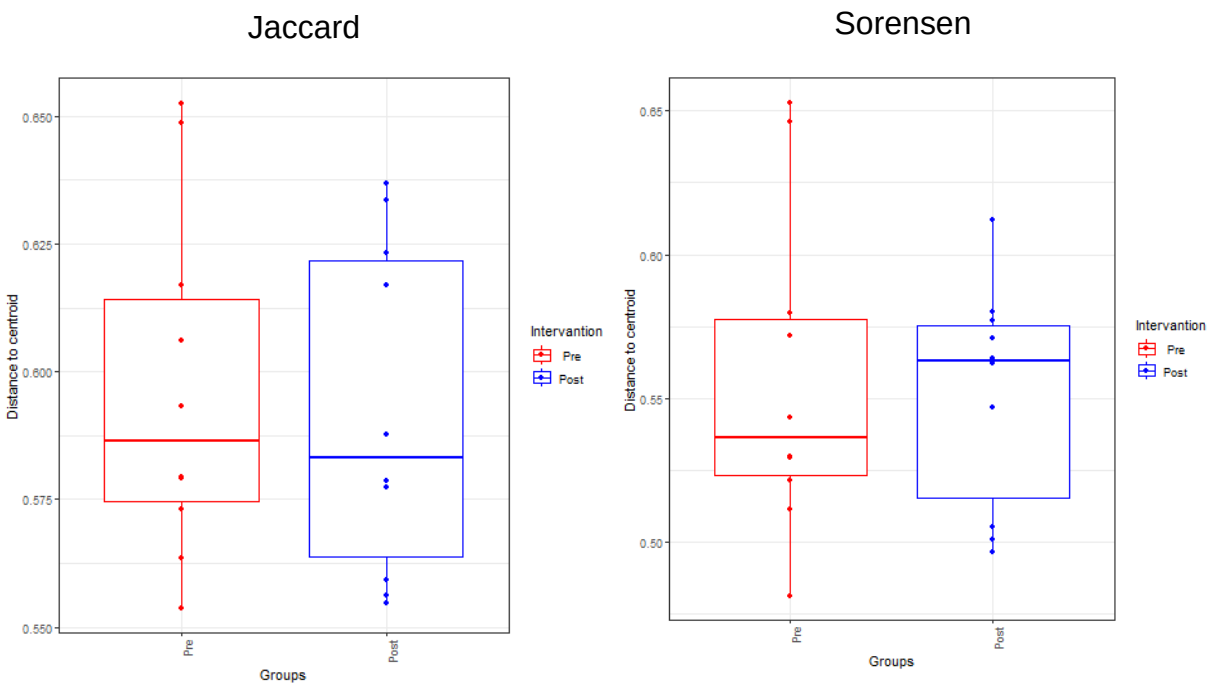


n= 6 (24 Samples)

Figure 28: Beta diversity in patients with PAD after short-term IGIV.

Analysis was done to compare pre- vs. post-intervention with vitamins supplements in the subgroup of patients that were in short-term IGIV. The results were presented in median quartiles and range. The comparison was made by using *t*-test. Any box plot shows significant *p*-value, is marked as *** <0.001, ** <0.01, * <0.05.

Results Summary: There is no significant difference in the observed diversity. The Post samples diverse almost equal to Pre samples diverse. In reviewing the medical and blood tests as well as procedures of stool collection for the outliers, no explanation could be identified.



n=4 (20 Samples)

Figure 29: Beta diversity in patients with PAD after long-term IGIV.

Analysis was done to compare pre- vs. post-intervention with vitamins supplements in the subgroup of patients that were in long-term IGIV. The results were presented in median quartiles and range. The comparison was made by using *t*-test. Any box plot shows significant *p*-value, is marked as *** <0.001, ** <0.01, * <0.05.

Results Summary: There is no significant difference in the observed diversity. The Post samples were more diverse than the Pre samples. More diverse meaning more targets detected

4.5.7. Summary of microbiome results

There were differences in the family targets of the gut microbiome between the different samples before and after interventions with some emerging and some disappearing. However, the gut microbiome was not able to show a significant difference in alpha and beta diversity. Further bigger sample size may be needed in the future to identify the potentially present differences. Also, utilising different microbial sequencing methods particularly by using metagenomic sequencing may provide more detailed and different results. Further analysis using different sequencing methodology and bigger sample size may be planned for the future microbiome analysis.

4.6. Results: miRNA Analysis

4.6.1. Introduction

Transcriptome Analysis Console (TAC) Software was the program utilized to run miRNA analysis following running the samples on the microarray's plates.

Adapted from ThermoFisher Scientific website; accessed 1/4/2019

(https://www.thermofisher.com/qa/en/home/life-science/microarray-analysis/microarray-analysis-instruments-software-services/microarray-analysis-software/affymetrix-transcriptome-analysis-console-software.html)

microRNA (miRs) regulate more than 60% of human protein-coding genes[112]. Also, miRs not only grant precision to protein expression[113], but may have many regulatory roles in various cellular processes and implicated in various diseases[114]. Circulating miRs account for over 100 and they may represent potential biomarkers of disease[115]. Serum or plasma miRs have been studied in cancer patients and their expression were up- or dysregulated compared to healthy subjects. miRNAs expression is dysregulated in human cancer through various mechanisms. Also, miRNAs may regulate the development of human tumours by acting as tumour suppressors or oncogenes[116.] Hornick suggested utilising circulating exosome miRs as novel minimally invasive biomarkers for acute myeloid leukaemia recurrence[117].

Nutrition is another identified factor that has complex relationship to miRs and gene targets. Macro- and micronutrients as well as newly identified dietary miRs may all participate in human diseases, its recovery and the healing process. Dietary miR database reported miRs in 15 dietary resources[38, 112]. Both endogenous mirRs and those derived from the diet have been expected to play a role in health and disease, particularly with regard to immune cell functioning during chronic inflammation and the fact that endogenous synthesis is altered by bioactive compounds in the

diet[112]. To the best of our knowledge, no data could be retrieved from the literature about circulating exosomal miR nor dietary effects on miR in PIDDs.

This section of the study aimed to elaborate some of this complex relationship between circulating exosome miRs and dietary intervention and to find out any future potentials in the clinical practice for the patients suffering from B-cell immunodeficiency.

4.6.2. Genes of interest in PAD

In less than 10% of CVID patients, the disease is familial and may be attributed to monogenic defects with a predominantly autosomal dominant inheritance[118-119].

4.6.2.1. Reported monogenic genes in CVID: Total of 16 genes

From the OMIM database: 13 genes: ICOS, TNFRSF13B, TNFRSF13C, CD19, CR2, MS4A1, CD81, IL21, LRBA, NFKB1, NFKB2, IRF2BP2, IKZF1[119-120,] We removed PRKCD gene that was described before[119] from this list and replaced by IRF2BP2[121]. PRKCD was reported as a new defect for CVID[122] (Previously labelled in OMIM as CVID, 9)[120] associated with autoimmunity. Many authors continue to cite this gene under CVID. This was later removed and corrected following Oliveira's[123] review, showing that the picture was more towards Autoimmune Lymphoproliferative disorders (ALPS) rather than CVID.

4.6.2.2. Genes reported in the literature to be associated with CVID

The functions are mainly related to T-cell and B-cell defects leading to a deficiency in antibody production (Not reported in OMIM): 3 genes: CTLA4 and PLCG2 (Both cause a similar phenotype or modify the severity of the disease with comorbidities) and MSH5 (Regulation of Ig class switch recombination[119].

4.6.2.3. Extra genes that may be related to CVID: with OMIM number

TNFSF12 (TWEAK) *602695, TNFRSF7 (CD27) *186711, IL21R *605383, PIK3CD *602839, PIK3R1 *171833, VAV1 *164875, RAC2 *602049, BLK *191305 2, PRKCD ***** 176977[120].

4.6.2.4. Genes related to IgA deficiency

IGAD1 *% 137100[120].

4.6.2.5. Dietary resources for miRNAs[124]

15 dietary products were found to have miRNAs that can be present in the human sera.

Types	Species
Animal	Human Breastmilk
	Cow milk
	Cow Fat
	Atlantic Salmon
	Chicken
	Pig
Plant	Apple
	Banana
	Corn
	Grape
	Orange
	Rice
	Soybean
	Tomato
	Wheat

Table 24: List of miRNAs identified in food components.

The different types and species of dietary microRNA where mature and precursor miRNAs have been found based on Dietary MicroRNA Database (DMD).

(*Note: adapted from Chiang et al. 2015*[124])

4.6.3. miRNA Analysis Plan

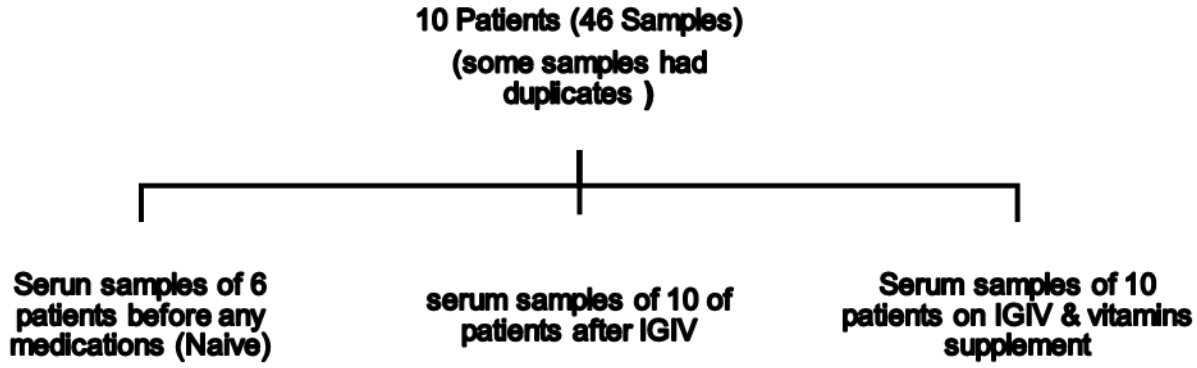


Figure 30: Analytical plan for miRNA.

Blood samples were collected from 6 patients who were naïve (never received any IGIV) and 4 who had been on IGIV for a long time (since childhood). Second sample sets were collected from all participants at 8 weeks on intervention and 6-week washout period (off intervention).

Patient number	Condition			Duplicate			Total
	Pre IgG-administration (naïve)	Post IgG-infusion/ pre-vitamins	Post combined IgG and vitamins	Pre IgG-administration (naïve)	Post IgG-infusion/ pre-vitamins	Post combined IgG and vitamins	
P1	P1a_PreIg	P1a_PreVit	P1a_PostVit	P1b_PreIg	P1b_PreVit	P1b_PostVit	6
P2	P2a_PreIg	P2a_PreVit	P2a_PostVit	P2b_PreIg	P2b_PreVit	P2b_PostVit	6
P3	P3a_PreIg	P3a_PreVit	P3a_PostVit	P3b_PreIg	P3b_PreVit	P3b_PostVit	6
P4	P4a_PreIg	P4a_PreVit	P4a_PostVit	P4b_PreIg			4
P5	P5a_PreIg	P5a_PreVit	P5a_PostVit	P5b_PreIg	P5b_PreVit	P5b_PostVit	6
P6	P6a_PreIg	P6a_PreVit	P6a_PostVit	P6b_PreIg	P5b_PreVit	P5b_PostVit	6
P7		P7a_PreVit	P7a_PostVit		P7b_PreVit	P7b_PostVit	4
P8		P8a_PreVit	P8a_PostVit		P8b_PreVit	P8b_PostVit	4
P9		P9a_PreVit	P9a_PostVit				2
P10		P10a_PreVit	P10a_PostVit				2
RNA -C	2						2
Total	6	10	10	6	7	7	48

Table 25: miRNA conditions on the microarray.

Ten samples were collected after IGIV and 10 after both IGIV and vitamin supplementation (7 had duplicates on microarray). Six samples were collected from patients at naïve status before any medications (5 had duplicates on the microarray).

4.6.4. Quality assessment of microRNA (Agilent readings)

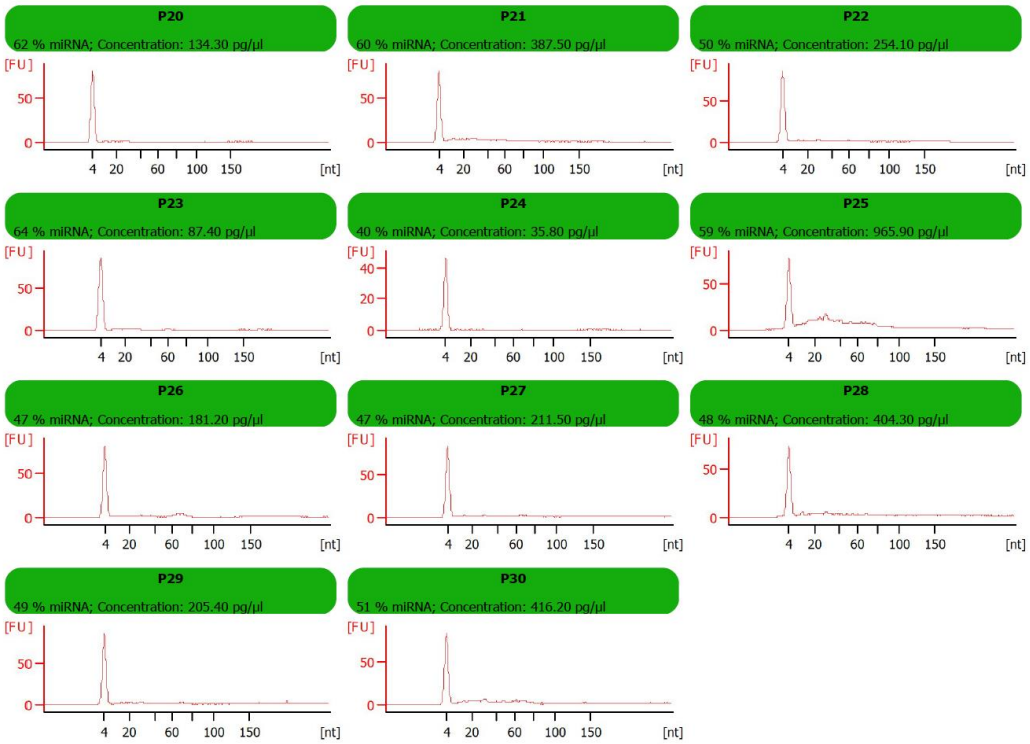


Figure 31: Quality assessment of miRNA using Agilent bioanalyser.

This chip offers one of the few possibilities to quantify miRNA. Fragments with a size of 15–40 nt were defined as miRNA. The concentration of miRNA was calculated as absolute amount [pg] and as a percentage of small RNA [%]. Please refer to Chapter 2 (section 2.2.2.4.1).

The outcome results of extracted miRNA from the Genetitan were analysed by Transcriptome Analysis Console (TAC 4.0.1) Software program for miRNA gene expression.

4.6.5. Summary of the results of miRNAs

Comparison	Group 1	Group 2	Count 1	Count 2	Up	Down
PostVit vs. PreIg	PostVit	PreIg	17	12	40	8
PostVit vs. PreVit	PostVit	PreVit	17	17	2	1
PreIg vs. PreVit	PreIg	PreVit	12	17	2	18

Table 26: Summary of miRNAs that were altered.

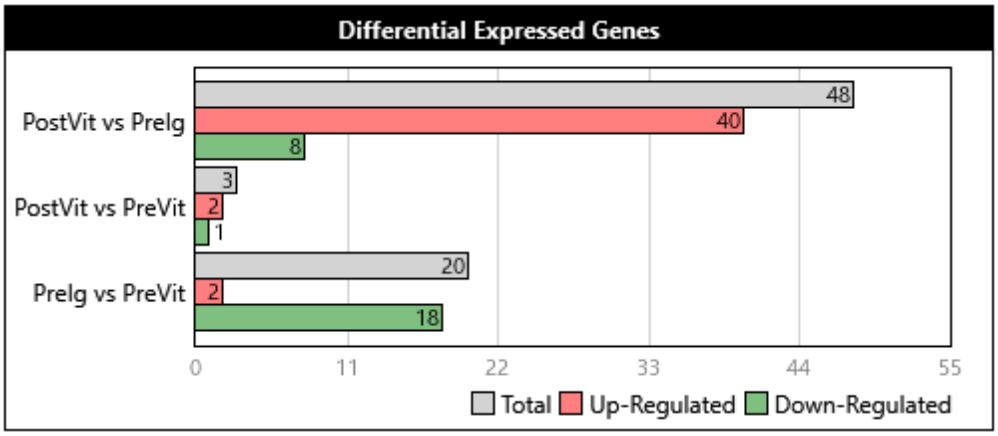
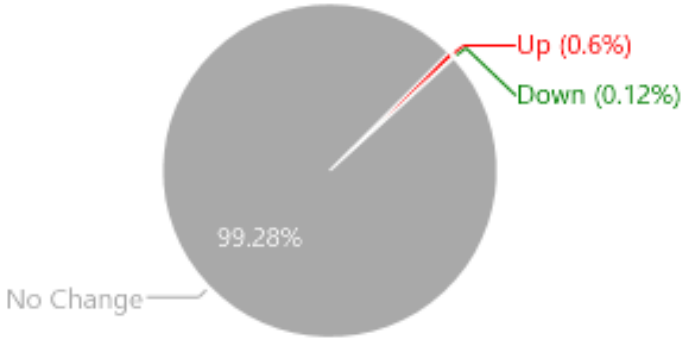


Figure 32: Summary of miRNAs that were altered.

PostVit vs. PreIg

• PostVit: 17 samples, PreIg: 12 samples



Filter criteria:

• Fold Change: > 2 or < -2

• P-val: < 0.05

Total number of genes: 6631

• Genes passed filter criteria: 48 (0.72%)

• Up-Regulated: 40 (83.33%)

• Down-Regulated: 8 (16.67%)

4.6.6. Report of the miRNAs that were dys- and up-regulated

miRNA	Post-Vit Avg (log2)	Pre-Ig Avg (log2)	Post-Vit SD	Pre-Ig SD	Fold Change	P-val	FDR P-val	Chromosome
hsa-miR-4440	2.68	4.87	1.77	1.33	-4.56	0.002	0.364	chr2
hsa-miR-3148	1.94	3.65	1.13	0.91	-3.28	0.002	0.364	chr8
hsa-miR-4793-3p	2.38	4.22	1.49	1.6	-3.59	0.01	0.492	chr3
hsa-miR-4701-3p	0.53	1.72	0.78	2.1	-2.27	0.03	0.586	chr12
hsa-miR-595	0.4	2.07	1.07	1.86	-3.2	0.03	0.595	chr7
hsa-miR-185-3p	0.27	2.2	1.22	1.7	-3.79	0.04	0.623	chr22
hsa-miR-1268b	0.42	1.49	0.94	1.62	-2.09	0.04	0.646	chr17
hsa-miR-4763-3p	1.18	2.5	1.12	1.01	-2.5	0.04	0.648	chr22

Table 27: Dysregulated miRNAs.

Eight miRNAs showed dysregulation after vitamin supplementation when compared to condition before IGIV. None of the miRNAs showed statistical significance after correction of the *p*-value (FDR *p*-value).

miRNA	PostVit Avg (log2)	PreIg Avg (log2)	PostVit SD	PreIg SD	Fold Change	P-val	FDR P-val	Chromosome
hsa-miR-4497	7.16	3.01	0.66	2.55	17.75	1.71E-08	0.0001	chr12
hsa-miR-122-5p	6.64	2.13	1.49	2.36	22.91	7.08E-06	0.0235	chr18
hsa-miR-320d	5.61	3.39	1.12	1.82	4.67	3.44E-05	0.0723	chr13
hsa-miR-320c	7.28	5.59	0.74	1.22	3.24	4.36E-05	0.0723	chr18
hsa-miR-4429	4.62	2.37	1.53	1.12	4.78	0.0003	0.2093	chr2
hsa-miR-320b	7.41	6.11	0.73	1.67	2.47	0.0005	0.2093	chr1
hsa-miR-451a	6.34	3.76	1.49	1.45	6	0.0005	0.2093	chr17
hsa-miR-92b-3p	4	1.17	1.87	1.32	7.1	0.0005	0.2161	chr1
hsa-miR-320a	7.48	6.26	0.74	1.29	2.34	0.0007	0.248	chr8
hsa-miR-486-5p	11.21	9.22	1.41	1.01	3.98	0.0009	0.2931	chr8
hsa-miR-30d-5p	3.66	0.59	1.95	1.18	8.35	0.0014	0.3211	chr8
hsa-let-7b-5p	8.12	7.06	1	0.67	2.09	0.0015	0.3489	chr22
hsa-miR-92a-3p	10.75	9.42	1.23	0.83	2.52	0.0016	0.3613	chr13
hsa-miR-4745-5p	2.22	0.2	1.71	0.86	4.05	0.0021	0.3642	chr19
hsa-miR-22-3p	3.9	0.98	1.93	1.88	7.57	0.0025	0.3848	chr17
hsa-miR-140-3p	5.29	1.86	1.89	1.71	10.82	0.0026	0.3848	chr16
hsa-miR-150-5p	2.93	1.69	1.52	0.57	2.37	0.0029	0.4072	chr19
hsa-miR-193a-5p	3.75	1.16	1.51	1.14	6	0.0031	0.408	chr17
hsa-miR-3135b	2.59	0.39	1.97	0.98	4.61	0.0032	0.408	chr6
hsa-let-7c-5p	6.35	5.2	1.41	1.39	2.22	0.0046	0.4121	chr21
hsa-miR-25-3p	5.98	3.53	1.73	1.3	5.49	0.0068	0.4359	chr7
hsa-miR-194-5p	1.7	0.36	1.67	0.35	2.54	0.0072	0.4433	chr1
hsa-miR-423-5p	5.25	2.35	2.03	1.89	7.48	0.0077	0.4528	chr17
hsa-miR-320e	1.85	0.59	1.85	0.33	2.39	0.0084	0.4649	chr19
hsa-miR-652-3p	2.14	0.49	2.1	0.51	3.14	0.0084	0.4649	chrX
hsa-miR-19b-3p	4.78	2.83	1.82	1.89	3.88	0.0106	0.5078	chr13
hsa-miR-222-3p	2.98	0.99	1.63	1.47	3.99	0.0118	0.5189	chrX
hsa-mir-484	2.34	1.06	1.65	0.42	2.42	0.014	0.5524	chr16
hsa-miR-484	2.07	0.55	1.95	0.61	2.87	0.0172	0.5856	chr16
hsa-miR-20b-5p	3.58	1.87	1.92	1.28	3.29	0.0193	0.5856	chrX
hsa-miR-197-3p	4.3	3.24	1.11	1.25	2.09	0.0207	0.5856	chr1
hsa-let-7i-5p	4.52	2.66	2.04	1.07	3.63	0.0207	0.5856	chr12
hsa-miR-106b-5p	4.96	3.65	1.41	1.36	2.49	0.0267	0.5856	chr7
hsa-miR-425-5p	5.16	3.78	2.02	1.7	2.6	0.0305	0.5948	chr3
hsa-miR-6125	5.12	3.61	1.44	0.98	2.85	0.0351	0.6231	chr12
hsa-miR-342-3p	4.02	2.35	1.67	1.45	3.17	0.0393	0.6394	chr14
hsa-miR-660-5p	1.87	0.57	1.42	0.93	2.47	0.0426	0.6505	chrX
hsa-miR-1246	4.74	2.12	1.57	0.98	6.16	0.0438	0.6551	chr2
hsa-miR-151a-3p	2.19	0.76	2.06	1.01	2.7	0.0486	0.6693	chr8
hsa-miR-4508	4.48	3	1.64	1.32	2.78	0.0488	0.6693	chr15

Table 28: Up-regulated miRNAs.

Only 2 showed statistical significance after correction of the *p*-value (FDR *p*-value).

40 miRNAs showed up-regulation after vitamin supplementation when compared to the condition before IGIV.

4.6.7. Volcano plot

The miRNAs were presented by the Volcano plot diagram that plots significance versus fold-change on the y- and x-axes, respectively.

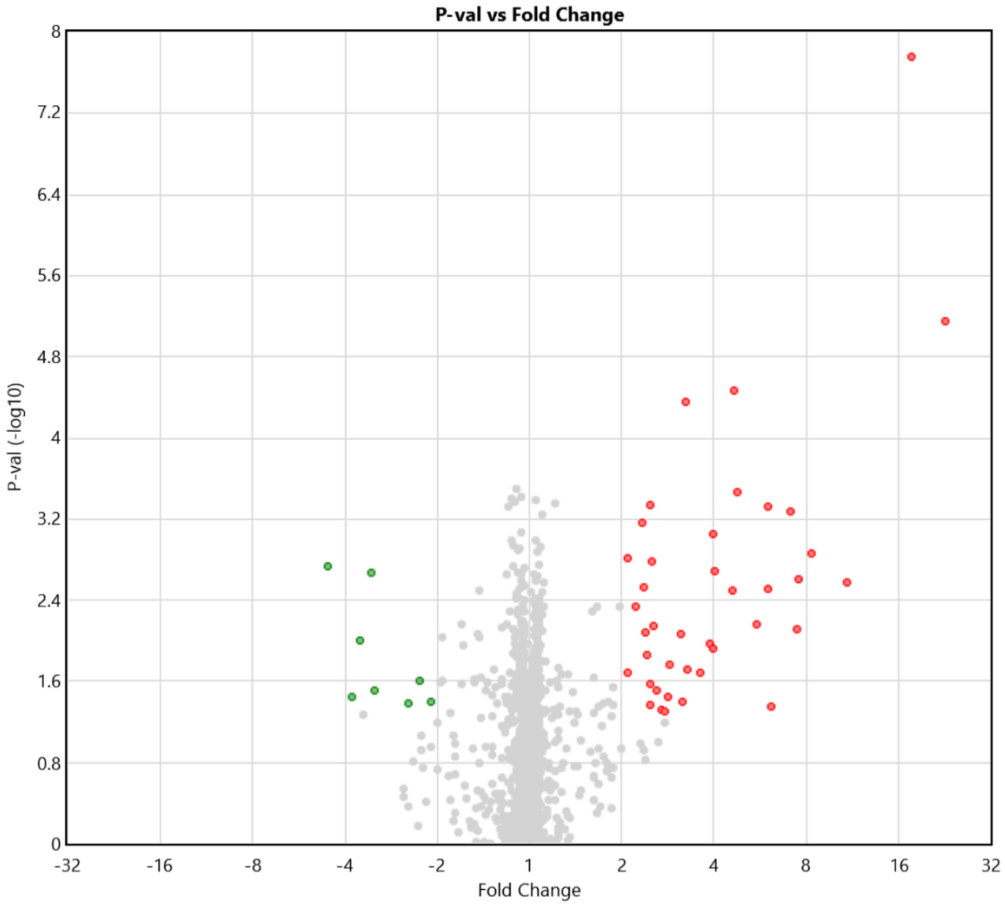


Figure 33: Volcano plot to quickly identify changes in large datasets.

The grey TCs were those filtered out by the table. The green TCs were down-regulated while the red TCs were represented as up-regulated. If we switch the condition pair or the filter criteria in the table, the data in the graph will change accordingly.

A volcano plot is a type of scatter plot. Volcano Plot graph plots significance versus fold-change on the y- and x-axes, respectively. The X-axis is the linear-fold change from the current condition pair; Y axis is -log10 *P*-value of the ANOVA *P*-values.

4.6.8. Venn Diagram

Only the comparison between samples in the Post-Vitamins and Pre-IGIV conditions showed miRNAs that were statistically significant after computing corrected *P*-value (FDR *P*-value).

Figure 34: Venn diagram to identify the intersection of genes between comparison(s).
Venn Diagrams based on Gene Lists was used to obtain common genes based on multiple analyses. The intersected Gene Lists appeared in the Venn diagram.

4.6.9. Serum miRNAs in PIDDs with statistical significance

miRNA	postvit Avg (log2)	preIg Avg (log2)	postvit SD	preIg SD	**Fold Change**	***P*-value**	**FDR *P*-value**	**Genes of interest**	Chromosome
hsa-miR-122-5p	7.16	3.01	0.66	2.55	17.75	1.71E-08	0.0001	CD19, IL21, PLCG2	chr18
hsa-mir-4497	6.64	2.13	1.49	2.36	22.91	7.08E-06	0.0235		chr12

Table 29: Up-regulated serum miRNAs in peripheral blood of PAD following intervention. Associated genes of interest in PAD (n=10) disease and to dietary changes were presented as well.

Up-regulated miRNA were compared further in a subset of patients (6 out of 10).

miRNA	PostVit Avg (log2)	PreIg Avg (log2)	PostVit SD	PreIg SD	Fold Change	*P*-value	FDR *P*-value	Chromosome
hsa-miR-4497	7.37	3.01	0.59	2.45	20.53	1.22E-06	0.0081	chr12

Table 30: Up-regulated serum miRNAs in peripheral blood of PAD.
This analysis was done following intervention in patients with similar clinical conditions regarding IGIV administration (6 patients only).

4.6.10. PCA mapping (Quality control view)

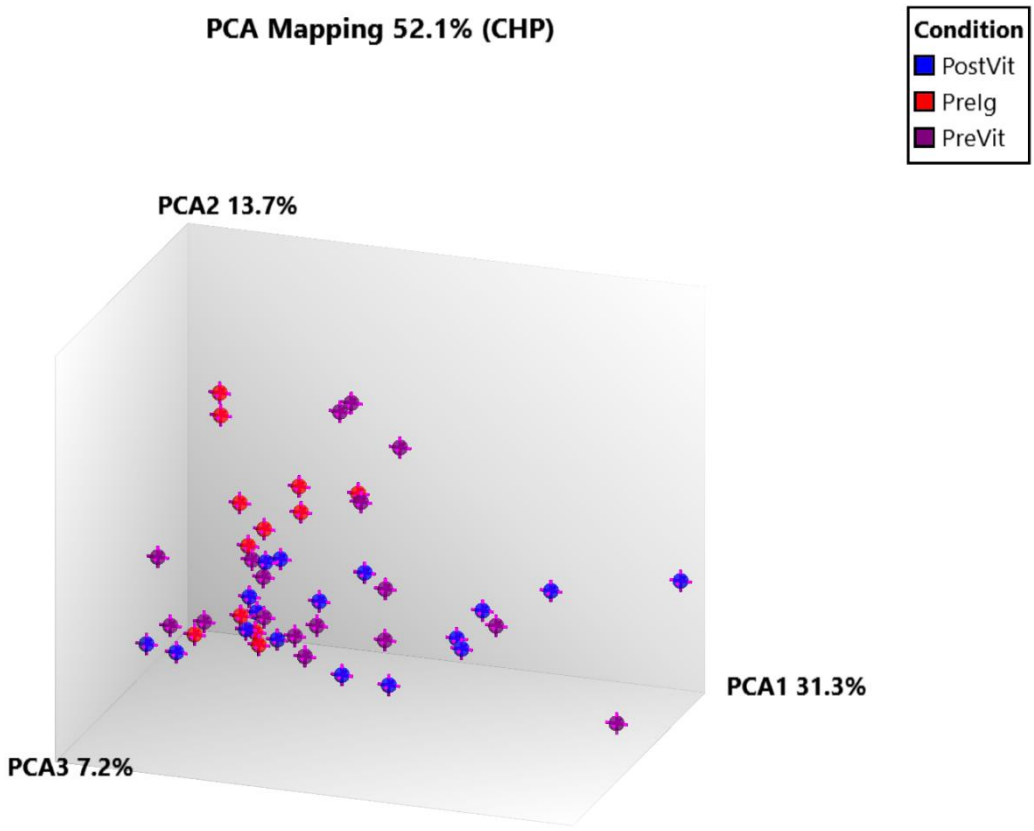


Figure 35: Principal Component Analysis (PCA) mapping.

PCA plot, coloured by a custom user attribute, computes a set of linearly uncorrelated variables each of which is an orthogonal linear combination of the gene components. These variables are sorted to have decreasing variance. Retaining the top few components yields a reduced dimensional view of the samples that represents most of the variance among the samples. PCA is simple to use and requires no control parameters.

4.6.11. Summary of the microRNA results

In the present study, the microRNA (miRNA) expression profiles of patients with PAD before and after intervention with micronutrients were captured using microRNA array plate. 2 miRs (hsa-miR-122-5p and hsa-mir-4497) had been identified to be up-regulated with the intervention step by multivitamin supplementation. Furthermore, bioinformatics analyses will be used to identify the genes and pathways, which were specifically associated with PAD and micronutrient intake-associated miRNAs. Furthermore, the results require validation by Real-Time PCR (RT-qPCR) analysis as part of the future plan.

4.7. Discussion

In the current study, a dietary change is suggested to play an immunomodulatory effect on the miRNA and microbiome on patients with PIDD particularly PAD. This theory was never been tested before in PIDD patients. Few reports about associations of miRs (alone or combined with microbiome) to dietary intervention in malignancy either for miRNA received medical interest in colorectal and oral cavity cancers[102,125].

Few studies have suggested that miRNAs play a role in the inter-domain interaction between the gut microbiome and host and that this has an effect on symbiosis as well as on dysbiosis and the occurrence of illnesses. Three theories have been postulated which may explain, in part, this communication between the host, gut microbiome, and miRNAs; these are: (i) host gene expression is regulated by miRNAs, (ii) host miRNAs are influenced by gut microbiota, and (iii) the host miRNAs influences the gut microbiota[126].

In our study, the relationship between dietary intervention and the microbiome was not clear by alpha and beta diversities. However, the individual families showed some changes where some lost and some showed up after intervention. These changes in the detected families was not associated with any change in bowel behaviour or other symptoms. Any associated significance at this point could not be identified (outside the scope of the study) mainly because of the sample small size (a larger size is usually needed to see changes in the microbiome). Also, the short duration of the interventional step (8 weeks), and absence of follow-up of the microbiome in the period after stopping the intervention may be contributing factors. In contrast, changes in the miRs were observed.

Based on the miRBase Registry; www.mirbase.org[127], around 1917 microRNAs have been identified in humans (homo sapiens). More than 2500 miRNAs have been described[126], and around 2300 true human mature miRNAs, 1115 of which are currently annotated in miRBase V22, were estimated as the total number of true human miRNAs[128]. In our study, the total number of identified miRs was 6631, of which, 48 passed the filter criteria (40 up-regulated and 8 down-regulated). Furthermore, two miRs (hsa-miR-122-5p and hsa-mir-4497) were identified to be up-regulated when intervention with both IGIV and diet was performed. These two miRs will be discussed further in Chapter 5. hsa-mir-4497 was found to be associated with some B cells of interest: CD19, IL21, and PLCG2. hsa-mir-4497 was reported as one of the B cell miRs when they performed deep sequencing of the small RNA transcriptome of normal and malignant human B cells[129].

There are several important limitations of this study that could be addressed in future research. First, the study is focused on micronutrients and IGIV effects. We used multiple vitamins which were added on top of the regimen of regular IGIV and we assumed a synergistic effect; however, this is not been tested. Second, the sample size is small, and while it may be enough for the proof of concept, the results cannot be generalised and a larger sample may be required for similar studies. Because this study included patients with rare disorders, involvement of multiple immunodeficiency point of care centres may help to increase the sample size. The third is the use of microarrays. Microarray technology may provide a great opportunity for clinical and research work; however, multiple backwashes must be considered. The access and cost that can limit the current use as clinical tests, the number and quality of RNA and miRNAs extracted per sample, the need for cellular disruption to reach the genetic materials of interest and the general limitation of each microarray which can only provide knowledge about the genes that are present on the array[130]. Huge scientific evidence supports the use of microarrays despite these limitations[88, 89, 125, 126, 129]. The final limitation is the short duration of the intervention step (8 weeks). Although it is not one of our main objectives, this duration is not sufficient to allow the observation of clinical improvement or worsening symptoms. All of these limitations do not undermine the quality and integrity of this research and the study was able to give some answers to the research question. The miRNAs identified in this study support the hypothesis of dietary influence on the complex communication between the gut microbiome and host via microRNAs and give a solid baseline for further workup and future investigation. Some direct beneficiary points that were performed but not discussed in this report include the fact that we identified some pathogenic organisms from

faecal samples using the microbiome arrays and we gave the patients the opportunity for a better clinical evaluation by requesting stool culture testing and providing proper antibiotics before the occurrence of clinical symptoms.

4.8. Summary

In this part of the study, although the gut microbiome did not reveal a significant difference in the diversity between the different intervention steps, the serum exosome microRNA uncovered two miRs which were up-regulated significantly after the interventions with vitamin supplementation and intravenous immunoglobulin administrations. One of these two miRs, hsa-miR-4497, was statistically significant, even when comparing the same conditions for the ten patients who were never exposed to IGIV before. hsa-miR-4497 was found to be associated with some genes of interest and this miR was reported previously with B cells[129.]

Further validation using RT-qPCR analysis, followed by bioinformatics analyses to identify the genes and pathways, which are specifically associated with PAD and micronutrient intake-associated miRNAs, is needed to confirm the current findings of microRNA signature changes and gene expression.

CHAPTER 5

DISCUSSION AND FUTURE PLANS

5.1. Introduction

CVID and SAD share a lot of clinical similarities, and almost all have recurrent infections ranging from mild to severe autoimmune conditions, allergy and malignancy, similar to other PIDDs. Patients with CVID, IgG subclass deficiencies, selective IgA deficiency (SIgAD) and SAD share mutations in modifying genes, like the transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) gene. Furthermore, particularly in CVID/SIgAD families[83], TACI has been suggested as a modifying gene which may be linked to states of defective class switch recombination, abortive plasma cell formation, and hypogammaglobulinaemia in response to BAFF and APRIL, which has received significant attention. TACI deficiency is also closely related to lymphoproliferation (like splenomegaly) and autoimmunity in mouse models[118,131-140]. Despite the focus of previous reports on proteotypic monogenic disorders with Mendelian inheritance, no unifying mechanism can explain the different spectrum of severity nor the correlation to clinical findings in patients with CVID and SAD. The most abundant group of PID patients (CVID and SAD) that received IUIS classification under the category of predominant antibody deficiency syndromes have mostly undefined genetic roots in more than 80% of patients.

To clarify the underlying mechanistic pathways and to search for more precise clinical and prognostic factors, we investigate the relationship between the triad (the immunomodulatory effect of micronutrients, circulating exosome miRNAs and gut microbiome).

5.2. Clinical Parameters

The first part of this study evaluated the current data of patients with PIDD to identify any clinically relevant factor that can help with the re-clustering and re-stratifying of patients with PIDD with the aim to improve patient care and identify monitoring markers for this chronic illness. The second part investigates the possibility of dietary intervention with micronutrients (vitamins A, E, B and D)

Common biomarkers continue to be of significant value in the clinical care of PIDD. The first was CRP, which is widely used in clinical practice. CRP is a homopentameric acute-phase inflammatory protein that was discovered in 1930 by Tillet and Francis during the working-up of patients suffering from *Pneumococcus* infection and it has a half-life of 19 hours[100]. Elevated CRP in this study was associated with bronchiectasis and no-allergy complications. This interesting observation of the results is logistical if we look at bronchiectasis as a process occurs in the context of chronic airway infection and inflammation[141]. Moreover, allergy in nature does not give rise to a CRP response[142]. The second inflammatory marker was WBC, which was high in patients without complications. This reverse relationship with WBC and bronchiectasis complications suggests that

bronchiectasis was higher in those whose body was unable to produce sufficient WBC to mount the infection thereby leading to increased infection and lung destruction (bronchiectasis). A study of 87 patients with bronchiectasis found significant associations between the HRCT bronchiectasis scores and elevated ESR, CRP, WBC and neutrophil count[143]. The third marker was the initial IgG level that were recorded in the clinics at establishing the diagnosis during first visits. The initial IgG levels were lower in the bronchiectasis complications and no-allergy complication group, indicating that the increased bronchiectasis complication was associated with a lower initial IgG. Also, this may suggest the level of confidence in the PIDD diagnosis without overlapping possibility for allergy based on the currently used international criteria in immunology clinics [83].

Another important factor that was found to be correlating to bronchiectasis diagnosis in PIDD patients was the age of presentation to a medical service. It seems that presentation earlier in life may suggest a more severe phenotype and therefore additional bronchiectasis complication.

5.3. Gut microbiome and miR signature in PAD

We proposed that change in diet alters the nutrients available to microbiota resulting in the growth of some species and a change of microbiota composition. We identified some families with changes, but no significant relationship between gut microbiome alpha and beta diversity and dietary changes were observed.

On the other hand, two miRs (hsa-miR-122-5p and hsa-mir-4497) were identified to be up-regulated when an intervention with both intravenous immunoglobulin administration and diet was completed.

hsa-miR-122-5p and hsa-mir-4497 in the literature

When Searching for miRs in the literature, around 169 sources were identified, including miRbase, GeneCard, EntrezGene, OMIM, HGNG, Mastermind and others (GeneCards)[144].

hsa-miR-122-5p (accession number of MIMAT0000421) is located on chromosome 18 and has a sequence of UGGAGUGUGACAAUGGUGUUUG[127]. MIR122 (MicroRNA 122) is an RNA Gene, and is affiliated with the miRNA class. Diseases associated with MIR122 include non-alcoholic fatty liver disease and hepatitis C. Among its related pathways are MicroRNAs in cancer. Also, interacting proteins for the miR122 gene were represented by Gene Ontology (GO) (Biological Process for MIR122 Gene) as gene silencing by miRNA (GO:0035195) (GeneCard)[144].

miR-122-5p has a tumour suppressor function through different molecular pathways. Seventy-one breast cancer patients were investigated to evaluate the expression profiles of miR-122-5p and its target ADAM10. ADAM10 expression was higher while miR-122-5p expression was lower in tumours than in normal tissue. It was proposed that miR-122-5p expression increases, especially in HER2+ cancer cells, to suppress ADAM10 shedding activity on HER2 receptors. miR-122-5p can function as a potential regulator of ADAM10 and help to overcome trastuzumab (monoclonal antibody used for breast cancer therapy) resistance and improve treatment outcome[145]. miR-122-5p has effects on the proliferation, cell cycle and apoptosis of human melanoma cell lines SK-MEL-110 and A375 and found that miR-122-5p expression is up-regulated in melanoma tissues. miR-122-5p inhibits the proliferation of SK-MEL-110 and A-375 cells possibly by affecting the cycle through NOP14 but has no effect on apoptosis[146]. miR-122-5p was also studied in 871 participants and found that the Levels of hsa-miR-122-5p and -885-5p were up-regulated in fatty livers and that both miRNA markers slightly improved the detection of fatty liver beyond the established risk factors. These miRNAs may be associated with fatty liver formation through the regulation of lipoprotein metabolism because hsa-miR-122-5p levels are associated with small VLDL, IDL, and large LDL lipoprotein subclass components[147]. Table 3 in Chapter 1 summarised some of the therapeutic development under clinical trials for miR-122 for hepatitis C virus infection.

hsa-mir-4497 (accession number of MI0016859) is located on chromosome 12q24.11, and has a sequence of ACCUCCGGGACGGCUGGGCGCCGGCGGCCGGGAGAUCCGCGCUUCCUGAAUCCCGGCCGGCCCGCCCGGCGCCCGUCCGCCCGCGGGUC[127].

miR4497 (MicroRNA 4497) is an RNA Gene, and is affiliated with the miRNA class. Diseases associated with miR4497 include Scapuloperoneal Spinal Muscular Atrophy and Brachyolmia. (Gene cards) [144]. miR4497 expression was down-regulated in laryngeal squamous cell carcinoma (LSCC) tumour tissues and cell lines compared to the normal counterparts. Overexpression of miR-4497 inhibits proliferation and induces apoptosis of LSCC cells, while down-regulating anti-apoptotic Bcl-2 proteins. GBX2 is a direct target of miR-4497 when the mechanism was evaluated[148]. hsa-mir-4497 is considered one of the B cell miRs that were identified by deep sequencing of the small RNA transcriptome of normal and malignant human B cells[129].

5.4. Conclusion

As these were preliminary data, it is planned that by the end of this study we may reach an answer about the cross-talk between the triad that we hypothesised. Primary immunodeficiency disorders are currently clinically evaluated based on the single gene defect. We believe that there are potential areas for the discovery of the roles of other factors, like miRNA, diet immunomodulatory effects, and gut microbiota, which may influence the disease in these patients; this is an area that lends itself to personalised therapies, which can be enriched by the results from our study.

5.5. Limitations

Recruiting sufficient number of subjects with homogeneity in symptoms was a limiting factor in this study. Despite that, we believe that this was a reasonable number as the disease is considered rare. Furthermore, this research was planned as a proof of concept study. Depending on the results of this study, future multicentre collaborations may be needed to recruit sufficient numbers of patients for statistically significant results to inform detailed treatment modalities.

5.6. Long-term future direction

The current study aimed to re-stratify the patients with PIDD through the identification of novel markers or mediators in specific disease phenotypes (CVID and SAD). Further studies can follow this fundamental study, including functional research projects to look for further details about regulators, and can help to modify patient disease and the future developments of targeted or personalised therapies. In particular, miRNA-antagonised insertion or silencing with or without exosome transferring or transfection can function as cargo delivery to other mediators in these patients. Gut microbiome modification or transplantation is also another area of interest for many types of research that can be evaluated to help patients with PAD.

5.7. Future Studies

Further validation using RT-qPCR analysis on the current results and findings followed by bioinformatics analyses to identify the genes and pathways, which were specifically associated with PAD and micronutrient intake-associated miRNAs will be carried out to confirm microRNA signature changes and gene expression. Moreover, additional studies with larger sample sizes with multicentre involvement will be performed.

CHAPTER 6

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APPENDICES

APPENDIX I- The budget sheet summary & Requested Materials and Consumables

The budget sheet summary.

Budget Category	2018	Total Cost
Travel & Conferences	2,000	2,000
Materials & Consumables	153,504	153,504
Total Cost	155,504	155,504

The amount was in Qatari Riyals (around 34,421.58GBP).

Details of requested materials and consumables for the research project.

Research Institution : Hamad Medical Corporation (HMC)

	Year	Budget Category	Budget Type	Description	Total Cost	Unit Cost	Quantity	Measure	Budget Interval	Budget Period	Justification
	2018	Travel & Conferences	Conference Sponsorship	010350/Dr.Maryam Ali Y Al-Nesf	2,000	500	4	Days	Yr	Y	attending conference related to the topic and presentation of work done in international conference.
	2018	Materials & Consumables	Laboratory Items	1 Kit Generation arrays for microbiome	78,466	78,466	1		Yr	Y	Laboratory array for microbiome needed for conducting the lab work
	2018	Materials & Consumables	Laboratory Items	Extraction Kits for microbiome, RNA, exome and peripheral blood for each kit	20,000	5,000	4		Yr	Y	Extraction kits for microbiome, RNA, exosome and peripheral blood (each kit has a cost of approximately 5000 QR)
	2018	Materials & Consumables	Laboratory Items	2 Kit of 24 arrays for the mRNA (part of the whole transcriptome)	55,038	55,038	1		Yr	Y	Budget is needed to conduct laboratory work of the research (part of the transcriptome arrays). The price is for the total set of the two kits arrays (total 48 arrays)

Total For Hamad Medical Corporation (HMC): 155,504

APPENDIX II- Ethical Approval

Ethical Approval.

مؤسسة حمد الطبية
Hamad Medical Corporation
HEALTH · EDUCATION · RESEARCH صحة · تعليم · بحوث

Sincerely,

Chairman of Institutional Review Board: _____

Signature: 

Date: _____

List of Approved Documents:

DOCUMENTTYPE	DOCUMENTNAME	LANGUAGE	NOOFPAGES	VERSIONNO
Recruitment Material	MRC-01-17-029_RecruitmentMaterial_Eng_V1.0_07-DEC-17_2Pages_159006.1_07-DEC-17_2Pages_159006.pdf	English	2	V1.0
Assent Form	MRC-01-17-029_AssentForm_Ara_V1.0_07-DEC-17_1Pages_159246.1_07-DEC-17_1Pages_159246.pdf	Arabic	1	V1.0
Assent Form	MRC-01-17-029_AssentForm_Eng_V1.0_07-DEC-17_1Pages_159269.1_07-DEC-17_1Pages_159269.pdf	English	1	V1.0
Data Collection Sheet	MRC-01-17-029_DataCollectionSheet_Eng_07-DEC-17_3Pages_159280.pdf	English	3	V1.0
Research Consent Form	MRC-01-17-029_ResearchConsentForm_Ara_V1.0_07-DEC-17_7Pages_159282.1_07-DEC-17_7Pages_159282.pdf	Arabic	7	V1.0
Research Consent Form	MRC-01-17-029_ResearchConsentForm_Eng_V1.0_07-DEC-17_7Pages_159285.1_07-DEC-17_7Pages_159285.pdf	English	7	V1.0
Interview/ Script	MRC-01-17-029_Interview/Script_Ara_V1.0_07-DEC-17_5Pages_159288.1_07-DEC-17_5Pages_159288.pdf	Arabic	5	V1.0
Interview/ Script	MRC-01-17-029_Interview/Script_Eng_V1.0_07-DEC-17_5Pages_159291.1_07-DEC-17_5Pages_159291.pdf	English	5	V1.0
Research Protocol	MRC-01-17-029_ResearchProtocol_V1.0_07-DEC-17_13Pages_159350.1_07-DEC-17_13Pages_159350.pdf	English	13	V1.0
Questionnaire/ Survey	MRC-01-17-029_Questionnaire/Survey_Ara_V1.0_07-DEC-17_4Pages_159365.1_07-DEC-17_4Pages_159365.pdf	Arabic	4	V1.0
Questionnaire/ Survey	MRC-01-17-029_Questionnaire/Survey_Eng_V1.0_07-DEC-17_3Pages_159378.1_07-DEC-17_3Pages_159378.pdf	English	3	V1.0
Other/Supporting document	MRC-01-17-029_MTTCellProliferationAssay_Eng_06Pages.pdf	English	6	V1.0

APPENDIX III- Food frequency questionnaire

The Dietary Questionnaire modified from the Harvard Food Frequency Questionnaire (with addition of traditional Arabic food). Arabic translation was performed for Non-English speakers. Reference: https://regepi.bwh.harvard.edu/health/FFQ/files/80out.pdf

Face to face Dietary Interview

What Have You Been Eating Lately? During the past 4 weeks, how often did you eat a serving of each of the foods listed here?

What is your AGE? 10 15 11 16 12 17 13 18 14 more Specify:	1. Are you: Female Male	2. Your Height	3. Your Weight (Kg)
4. Do you now take vitamins (like Flintstones, Centrum, Centrum Kids)? Yes No Please Specify brand:		5. How many do you take per week?	1–2 3–5 6–9 10 or more
6. Do you take any other separate vitamin or mineral pills? (NOT the multivitamin pill listed in question 4	Yes No	If yes, do you take any of the following? Vitamin A Fish Oil Vitamin E Iron Vitamin B Calcium supplement Vitamin C Other please specify: Vitamin D	
7. How often do you eat food that is fried at home, like fried chicken? 7.	Never/< 1/ week 1–3 times per week 4–6 times per week Daily	8. How often do you eat fried food away from home (like French fries, chicken nuggets)?	Never/< 1/ week 1–3 times per week 4–6 times per week Daily
If yes, what type of fat do you use to fry at home: butter coconut oil margarine corn oil crisco canola oil olive oil Palm oil Ghee (veg) Soy oil Sesame oil Sunflower oil other vegetable oil:		What type of margarine do you usually use: stick tub squeeze DON'T USE MARGARINE	
If you eat cold breakfast cereal, what type: high fiber (eg. All Bran) other (eg. Corn Flakes)			
What type of bread do you usually eat: Lebanese Bread whole wheat or dark bread Chapati/ Papard (Parata) Egyptian Bread white bread Iranian (Tannor) Rakak bread DON'T EAT BREAD Others; specify:			
Do your family bake cookies, cake or pies at home? No Yes		If yes, how often do you eat home-baked cookies, cake, or pies: Every day 1–3 times/ week 4–6 times/ week Less than one time/ week	
What kind of milk do you usually drink? (Check one) Whole Chocolate milk 1% Skimmed 2% Oher, Specify:			

	last 4 weeks		each week			each day			
Number of times	0	1–3	1	2–4	5–6	1	2–3	4–5	6+
Milk (buffalo, cow, camel, sheep, goat)									
Hot chocolate									
Cheese, plain or in sandwiches									
Cheese (cream, Akkiwi, haloom, Feta, white)									
Yogurt (plain, flavoured)									
Ice cream (cones, sandwiches, sundaes), plain/ Flavoured									
Milk Pudding									
Dream topping, milk made									
Kishta									
Labneh									

	last 4 weeks		each week			each day			
Number of times	0	1–3	1	2–4	5–6	1	2–3	4–5	6+
Orange juice or grapefruit juice									
Other juice									
Fruit drinks (Hi-C, Kool-aid, lemonade, sportsdrink)									
Melon									
Nabak									
Fruit cocktail, mixed fruit									
Orange or grapefruit									
Papya, Pear, Plum, Pomegranate, quince									
Mango									
Strawberries									
Melon/ watermelon									
Pineapple									
Raisins or prunes									
Dates (fresh or dried)									
Dried Fruits (apricot, Banana, mixed, Fig									
Blackberry, Apricot, Apple, Cherry, blackberry, banana, Grapes, Guava, Peaches									
Lemon									
Tamarind									
Canned fruits									

	last 4 weeks		each week			each day			
Number of times	0	1–3	1	2–4	5–6	1	2–3	4–5	6+
Corn									
Peas									
Tomatoes, tomato sauce, salsa									
Peppers (green, red or hot)									
Carrots									
Broccoli									
Green beans									
Baked, canned beans									
Agar, dried									
artichoke									
asparagus									
Avocado									
celery									
Beetroot/ Turnip									
Thyme									
Fennel									
Onion raw									
Grape leaves (with rice)									
Spinach									
mushrooms									
Radish (raw leave, raw root, white)									
garlic									
Coriander/ basil/ mint/ fenugreek/ parsley									
Brussels sprouts									
Squash, orange, winter or summer									
Spinach Greens (mustard, turnip, kale)									
Mixed vegetables									
Zucchini, yellow squash									
French fries, fried potatoes, tater tots									
Potatoes (baked, boiled, or mashed)									
Sweet potatoes or yams									
Cabbage, coleslaw or cauliflower									
Okra									
eggplant									
cucumber									
Lettuce salad									
Salad dressing									
Mayonnaise									

	last 4 weeks		each week			each day			
Number of times	0	1–3	1	2–4	5–6	1	2–3	4–5	6+
Barley									
Chips (potato, corn or others)									
Burghul (dark or light)									
Burr									
Rice (brown, basmati, Egyptian)									
Rice flour									
Rice pudding									
Semolina									
Parboiled Wheat									
Harees/ gireesh (wheatberries)									
Macaroni									
Noodles/ vermicelli									
Popcorn or corn									
Starch corn									
Crackers (cream or plain)									
Biscuits (digestive, date, gingernut or chocolate)									
coconut									
Nuts									
Seeds (melon seeds, pumpkin seeds, sesame, sunflower seeds, watermelon seeds									
Custard (canned or home made)									
Cookies or brownies									
Cake or cupcake/ Sponge cake									
Fruit cake/ Pancakes/ swiss rolls/ cheesecake									
Danish Pastries/ Doughnuts									
Pizza									
Dream									
Pie									
Jello									
Chocolate or candy bar									
Other candy (not chocolate)									
Coffee or tea (instant/ Arabic coffee)									
Soda, soft drink, pop (not sugar free)									
Soda, soft drink, pop (sugar free)									
Beer, wine, wine cooler, mixed drink or liquor									

	last 4 weeks		each week			each day			
Number of times	0	1–3	1	2–4	5–6	1	2–3	4–5	6+
Beans (baked, chili, or other), humus and Falafel (chickpea), foul (broad beans), others)									
Chickpea flour									
Beans (French/ blacked-eyed beans)									
lentils									
Mung beans seed									
Rice (kabsa, biryani)									
Spaghetti or other pasta (balaleet)									
Pizza									
Tacos, burritos									
Macaroni and cheese									
Hot dogs Sausage									
Hamburger (prepared anyway)									
Canned tuna									
Fried fish, fish sticks									
Other fish (Hammam, Kanad, Maid, Hamoor, Quraqufan, Safai, Shairy, Yanam									
Shellfish (Crabs, Shrimps, lobester)									
Cold cuts (baloney, ham, salami)									
Beef									
Camel meat									
Goat									
Brains, calf & sheep raw									
Chicken (Fried chicken, nuggets, boiled, roasted									
Turkey/ rabbit									
Roast beef or steak									
Liver, heart, kidney, tongue, spleen (organ meats)									
Peanut butter									
Bread (slice) toast, roll, or pita									
Butter (not margarine)									
Margarine									

	last 4 weeks		each week			each day			
Number of times	0	1–3	1	2–4	5–6	1	2–3	4–5	6+
Vegetable soup									
Other soup									
Cornbread or tortilla									
Eggs (yolk, white, hens)									
Bacon									
Hot cereal, grits									
Shawarma (Chicken or beef), Kofta, Tikkah, Kebab)									
Cold cereal									
Samosa, fried potato chops, bakeries									
Sweet roll or muffin									
Pancake, waffle, or french toast									
English muffin or bagel									
Biscuit									
Halawa Tehineh (Rahash)									
Marmalade									
Mixed Arabic sweets (eg. Baqlava, basboosa)									
Toffees mixed									

APPENDIX IV- Health & Clinical Stability assessment questionnaire

The Symptoms and Wellbeing Assessment Questionnaire (combined health quality SF6D and GHQ12)

SYMPTOMS SCORING:

KEY TO QUESTIONNAIRE Add individual scores and total each group. Add each group scores and give a grand total. • Optimal is less than 10 • Mild sickness: 10-50 • Moderate sickness: 50-100 • Severe sickness: over 100

DIGESTIVE TRACT
___ Nausea or vomiting
___ Diarrhea
___ Constipation
___ Bloated feeling
___ Belching, or passing gas
___ Heartburn
___ Intestinal/Stomach pain
Total _____

EARS
___ Itchy ears Total
___ Earaches, ear infections
___ Drainage from ear
___ Ringing in ears, hearing loss
Total _____

EMOTIONS
___ Mood swings
___ Anxiety, fear or nervousness
___ Anger, irritability, or aggressiveness
___ Depression
Total _____

ENERGY/ACTIVITY
___ Fatigue, sluggishness
___ Apathy, lethargy ___ Hyperactivity
___ Restlessness
Total _____

EYES
___ Watery or itchy eyes
___ Swollen, reddened or sticky eyelids
___ Bags or dark circles under eyes
___ Blurred or tunnel vision (does not include near- or far-sightedness)
Total _____

HEAD
___ Headaches
___ Faintness
___ Dizziness
___ Insomnia
Total _____

HEART
___ Irregular or skipped heartbeat
___ Rapid or pounding heartbeat
___ Chest pain
Total _____

JOINTS/MUSCLES
___ Pain or aches in joints
___ Arthritis
___ Stiffness or limitation of movement
___ Pain or aches in muscles
___ Feeling of weakness or tiredness
Total _____

LUNGS
___ Chest congestion
___ Asthma, bronchitis
___ Shortness of breath
___ Difficult breathing
___ cough
___ wheezing
Total _____

MIND
___ Poor memory
___ Confusion, poor comprehension
___ Poor concentration
___ Poor physical coordination
___ Difficulty in making decisions
___ Stuttering or stammering
___ Slurred speech
___ Learning disabilities Total _____

MOUTH/THROAT
___ Chronic coughing
___ Gagging, frequent need to clear throat
___ Sore throat, hoarseness, loss of voice
___ Swollen/discolored tongue, gum, lips
___ Canker sores
Total _____

NOSE
___ Stuffy nose
___ Sinus problems
___ Hay fever
___ Sneezing attacks
___ Excessive mucus formation
Total _____

SKIN
___ Acne
___ Hives, rashes, or dry skin
___ Hair loss
___ Flushing or hot flushes
___ Excessive sweating
Total _____

WEIGHT
___ Binge eating/drinking
___ Craving certain foods
___ Excessive weight
___ Compulsive eating
___ Water retention
___ Underweight
Total _____

OTHER
___ Frequent illness
___ Frequent or urgent urination
___ Genital itchor discharge
Total _____

GRAND TOTAL _____

SF-6D (Quality of life Questionnaire)

Physical functioning

1. Your health does not limit you in vigorous activities ☐
2. Your health limits you a little in vigorous activities ☐
3. Your health limits you a little in moderate activities ☐
4. Your health limits you a lot in moderate activities ☐
5. Your health limits you a little in bathing and dressing ☐
6. Your health limits you a lot in bathing and dressing ☐

Role limitation

1. You have no problems with your work or other regular daily activities as a result of your physical health or any emotional problems ☐
2. You are limited in the kind of work or other activities as a result of your physical health ☐
3. You accomplish less than you would like as a result of emotional problems ☐
4. You are limited in the kind of work or other activities as a result of your physical health and accomplish less than you would like as a result of emotional problems ☐

Social functioning

1. Your health limits your social activities none of the time ☐
2. Your health limits your social activities a little of the time ☐
3. Your health limits your social activities some of the time ☐
4. Your health limits your social activities most of the time ☐
5. Your health limits your social activities all of the time ☐

Pain

1. You have no pain ☐
2. You have pain, but it does not interfere with your normal work (both outside the home and housework) ☐
3. You have pain that interferes with your normal work (both outside the home and housework) a little bit ☐
4. You have pain that interferes with your normal work (both outside the home and housework) moderately ☐
5. You have pain that interferes with your normal work (both outside the home and housework) quite a bit ☐
6. You have pain that interferes with your normal work (both outside the home and housework) extremely ☐

Mental health

1. You feel tense or downhearted and low none of the time ☐
2. You feel tense or downhearted and low a little of the time ☐
3. You feel tense or downhearted and low some of the time ☐
4. You feel tense or downhearted and low most of the time ☐
5. You feel tense or downhearted and low all of the time ☐

Vitality

1. You have a lot of energy all of the time ☐
2. You have a lot of energy most of the time ☐
3. You have a lot of energy some of the time ☐
4. You have a lot of energy a little of the time ☐
5. You have a lot of energy none of the time ☐

GHQ-12 (Mental Health)

Have you recently?

1. Been able to concentrate on what you're doing?	**Better than usual**	**Same as usual**	**Less than usual**	**Much less than usual**
2. Lost much sleep over worry?	**Not at all**	**No more than usual**	**Rather more than usual**	**Much more than usual**
3. Felt you were playing a useful part in things?	**More so than usual**	**Same as usual**	**Less useful than usual**	**Much less useful**
4. Felt capable of making decisions about things?	**More so than usual**	**Same as usual**	**Less so than usual**	**Much less capable**
5. Felt constantly under strain?	**Not at all**	**No more than usual**	**Rather more than usual**	**Much more than usual**
6. Felt you couldn't overcome your difficulties?	**Not at all**	**No more than usual**	**Rather more than usual**	**Much more than usual**
7. Been able to enjoy your normal day-to-day activities?	**More so than usual**	**Same as usual**	**Less so than usual**	**Much less than usual**
8. Been able to face up to your problems?	**More so than usual**	**Same as usual**	**Less so than usual**	**Much less able**
9. Been feeling unhappy and depressed?	**Not at all**	**No more than usual**	**Rather more than usual**	**Much more than usual**
10. Been losing confidence in yourself?	**Not at all**	**No more than usual**	**Rather more than usual**	**Much more than usual**
11. Been thinking of yourself as a worthless person?	**Not at all**	**No more than usual**	**Rather more than usual**	**Much more than usual**
12. Been feeling reasonably happy, all things considered	**More so than usual**	**About same as usual**	**Less so than usual**	**Much less than usual;**

PHYSICIAN AND PATIENT GLOBAL ASSESSMENT

PtGA:

"In your experience, how severe is your disease?"

very mild	mild	moderate	severe	very severe

PhGA:

"In your opinion, compared to other patients with the same condition, how severe is the disease of patient X?"

very mild	mild	moderate	severe	very severe

DATA ANALYSIS

APPENDIX V- Supplementary file (Experimental trials)

1) Experiments performed to evaluate the best tests and kits to be utilised for the research project

2. Blood sample analysis (initial results for the first 6 samples of patients (before IGIV)

A. Total RNA

1. Quantitative analysis using the Nanodrop analyser

I. Samples collected into PAX tubes

		Nano drop							
#	Sample ID	Date and Time	Nucleic Acid Conc. ng/µl	A260	A280	260/280	260/230	Sample Type	Factor
Total RNA									
Pax (for whole blood cells)									
1	pax - p1	07/03/2018 09:55	184.5	4.612	2.202	2.09	1.96	RNA	40
2	pax - p2	07/03/2018 10:04	26.7	0.666	0.313	2.13	0.65	RNA	40
3	pax - p3	07/03/2018 10:05	27.9	0.697	0.314	2.22	1.72	RNA	40
4	pax - p3-2nd	07/03/2018 10:06	29.6	0.739	0.337	2.2	0.35	RNA	40
5	pax- p4	07/03/2018 10:07	0.7	0.018	-0.007	-2.63	0.01	RNA	40
6	pax- p5	07/03/2018 10:08	1.5	0.038	-0.001	-32.04	0.01	RNA	40

II. Serum tubes (exosome)

		Nano drop							
#	Sample ID	Date and Time	Nucleic Acid Conc. ng/µl	A260	A280	260/280	260/230	Sample Type	Factor
Total RNA									
Serum									
1	Exosom - p1A	07/03/2018 12:43	525.6	13.14	20.963	0.63	0.2	RNA	40
2	Exosom - p1B	07/03/2018 12:43	533.6	13.339	21.984	0.61	0.21	RNA	40
3	Exosom - p2A	07/03/2018 12:44	449.2	11.229	19.069	0.59	0.2	RNA	40
4	Exosom - p2B	07/03/2018 12:46	485.9	12.148	20.223	0.6	0.13	RNA	40
5	Exosom - p3A	07/03/2018 13:05	733.4	18.334	27.654	0.66	0.3	RNA	40
6	Exosom - p3B	07/03/2018 13:06	633.9	15.847	24.918	0.64	0.26	RNA	40
7	Exosom - p4A	07/03/2018 13:07	489	12.226	16.182	0.76	0.18	RNA	40
8	Exosom - p4B	07/03/2018 13:07	381.8	9.545	12.963	0.74	0.16	RNA	40
9	Exosom - p5A	07/03/2018 13:08	563.7	14.093	20.448	0.69	0.2	RNA	40
10	Exosom - p5B	07/03/2018 13:08	733.9	18.348	27.813	0.66	0.31	RNA	40
11	Exosom - p6A	07/03/2018 13:09	394.4	9.86	13.313	0.74	0.16	RNA	40
12	Exosom - p6B	07/03/2018 13:09	668.3	16.707	21.584	0.77	0.22	RNA	40

III. Buffy coat (EDTA tubes)

		Nano drop							
#	Sample ID	Date and Time	Nucleic Acid Conc. ng/µl	A260	A280	260/280	260/230	Sample Type	Factor
Total RNA									
Buffy coat (PBMC or lymphocytes)									
1	Trisol - p1	12/03/2018 12:34	1783	44.575	22.782	1.96	1.41	RNA	40
2	Trisol - p2	12/03/2018 12:34	865	21.626	13.004	1.66	0.35	RNA	40
3	Trisol - p3	12/03/2018 12:35	918.4	22.959	13.637	1.68	0.35	RNA	40
4	Trisol - p3-2nd	12/03/2018 12:36	594.2	14.855	9.458	1.57	0.24	RNA	40
5	Trisol - p4	12/03/2018 12:36	731.9	18.297	11.3	1.62	0.29	RNA	40
6	Trisol - p5	12/03/2018 12:37	1870.8	46.771	26.39	1.77	1.23	RNA	40
7	Trisol - p6	12/03/2018 12:37	1870.8	46.771	26.39	1.77	1.23	RNA	40
8	RE-TRI F - P1	12/03/2018 12:57	296.1	7.402	4.985	1.48	0.18	RNA	40
9	RE-TRI F - P2	12/03/2018 12:57	162.9	4.073	2.711	1.5	0.14	RNA	40
10	RE-TRI F - P3	12/03/2018 12:58	218.3	5.459	3.682	1.48	0.35	RNA	40
11	RE-TRI F - P4	12/03/2018 12:58	364.2	9.104	6.034	1.51	0.22	RNA	40
12	RE-TRI F - P5	12/03/2018 12:59	785.8	19.645	12.821	1.53	0.31	RNA	40
13	RE-TRI F - P6	12/03/2018 12:59	449.1	11.228	7.174	1.56	0.21	RNA	40

Interpretation:

RNA purity can be measured photometrically using the Nanodrop.

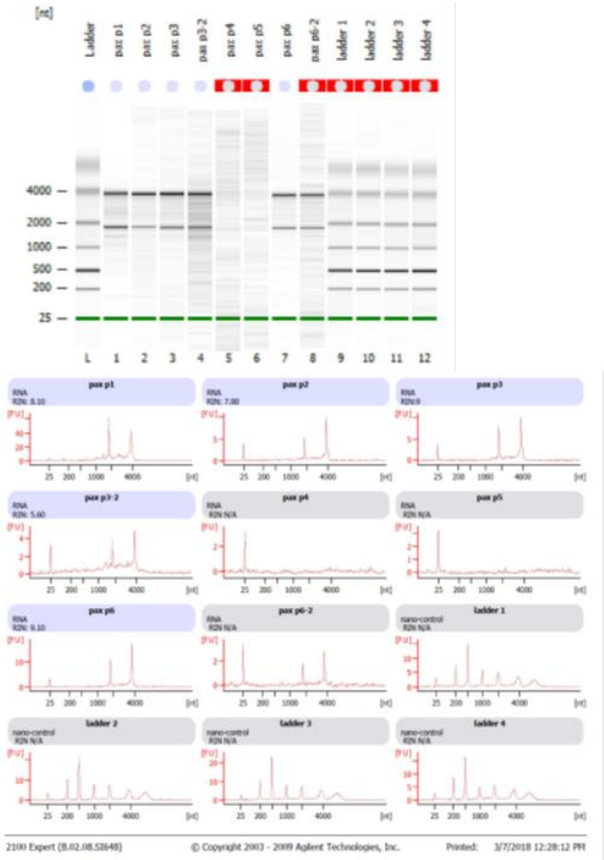
The optical density (OD) is measured at different wavelengths: 230 nm (absorption of contaminants and background absorption), 260 nm (absorption maxima of nucleic acids), and 280 nm (absorption maxima of proteins). The $OD_{260/280}$ ratio was used as an indicator of RNA purity. A ratio higher than 1.8 was assumed to be suitable for gene expression measurements.

For our samples, there was no consistency in the results to assess for purity correctly. This was observed on the same instrument by different students and staff in an anti-doping lab. Therefore, we will mainly use the Agilent Bioanalyser.

2. Qualitative analysis of total RNA using the Agilent Bioanalyser

I.PAX Tubes

		Agilent						Agilent reading: Fragment table for sample							
#	Sample ID	Date and time	RNA Area	RNA Concentration ng/µl	rRNA Ratio [28s / 18s]	RNA Integrity Number (RIN)	RIN	18S				28S			
								start size (nt)	End Size [nt]	Area	% of total Area	start size (nt)	End Size [nt]	Area	% of total Area
Total RNA															
Pax (for whole blood cells)															
1	pax - p1	07/03/2018 11:26	291.2	327	1.4	8.1 (B.02.08)	8.1	1,586	2,166	56.7	19.5	3,121	4,279	77.6	26.6
2	pax - p2	07/03/2018 11:26	35.2	40	2.5	7.8 (B.02.08)	7.8	1,770	1,933	3.4	9.7	3,677	4,092	8.6	24.3
3	pax - p3	07/03/2018 11:26	29.5	33	1.6	9 (B.02.08)	9	1,629	2,062	7.4	25.1	3,459	4,243	12.1	41
4	pax - p3-2nd	08/03/2018 11:26	39.1	44	1.5	5.6 (B.02.08)	5.6	1,753	1,923	2.4	6.1	3,678	4,087	3.7	9.4
5	pax- p4	09/03/2018 11:26	1.1	1	0	N/A (B.02.08)	N/A								
6	pax- p5	10/03/2018 11:26	8.8	10	0	N/A (B.02.08)	N/A								

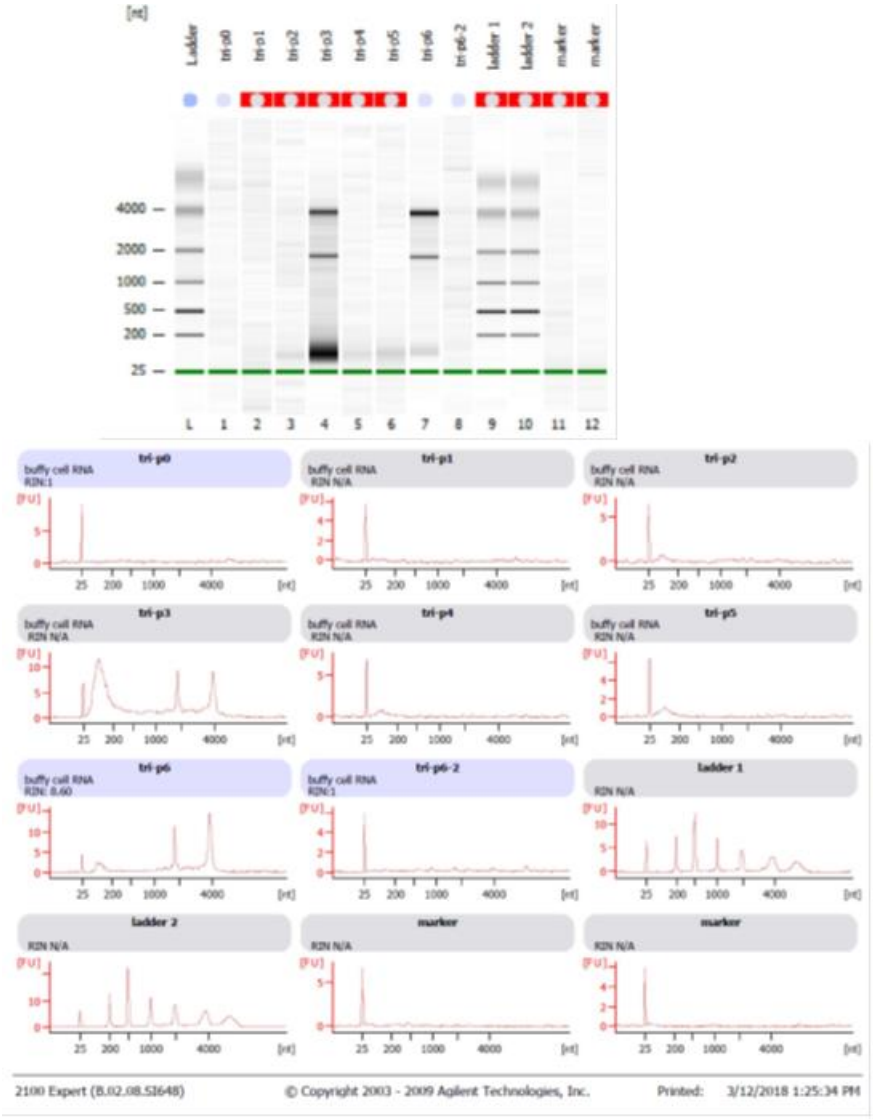


Interpretation: RNA integrities were assessed using the Agilent Bioanalyser 2100 which calculates RIN values of assayed RNAs (table and figure above). The RIN is the computational output of an algorithm which ranks several parameters obtained from the electropherograms, assigning a numerical value to RNA integrity.

The RNA integrity (RIN) for the first three samples (P1, P2 & P3) extracted using PAX tubes were above 7, indicating good quality total RNA.
The electropherograms for the total RNA present two distinct peaks (28S, and 18 S) in patient samples 1, 2, 3 and 6.

II.Buffy coat (EDTA)

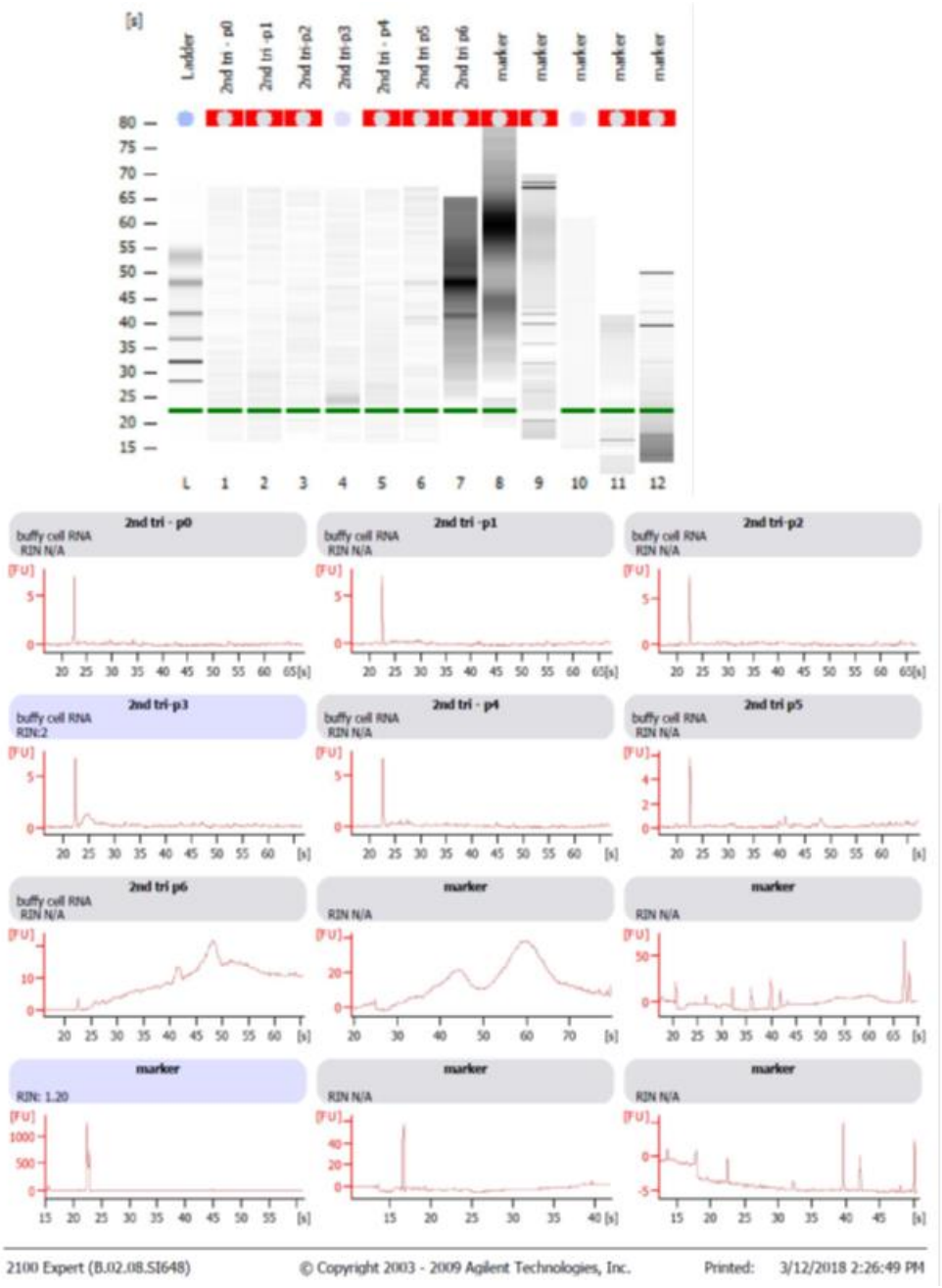
		Agilent						Agilent reading: Fragment table for sample							
#	Sample ID	Date and time	RNA Area	RNA Concentration ng/µl	rRNA Ratio [28s / 18s]	RNA Integrity Number (RIN)	RIN	18S				28S			
								start size (nt)	End Size [nt]	Area	% of total Area	start size (nt)	End Size [nt]	Area	% of total Area
Total RNA															
Buffy coat (PBMC or lymphocytes)															
1	Trisol - p1	12/03/2018 13:17	2.2	2	0	N/A (B.02.08)	N/A								
2	Trisol - p2	12/03/2018 13:17	3.3	3	0	N/A (B.02.08)	N/A								
3	Trisol - p3	12/03/2018 13:17	230.2	213	1.3	N/A (B.02.08)	N/A	1,558	2,054	11.2	4.9	3,477	4,564	15	6.8
4	Trisol - p3-2nd	13/03/2018 13:17	Not done												
5	Trisol - p4	14/03/2018 13:17	8.3	8	0	N/A (B.02.08)	N/A	2,337	2,591	0.3	3.6				
6	Trisol - p5	15/03/2018 13:17	9.3	9	0	N/A (B.02.08)	N/A								
7	Trisol - p6	16/03/2018 13:17	103	95	2.2	8.6 (B.02.08)	8.6	1,627	1,995	12.4	12.1	3,146	4,922	27.8	27
8	RE-TRIF - P1	17/03/2018 13:17	3.9	2	0	N/A (B.02.08)	N/A								
9	RE-TRIF - P2	18/03/2018 13:17	13.5	8	0	N/A (B.02.08)	N/A								
10	RE-TRIF - P3	19/03/2018 13:17	29.7	18	1	N/A (B.02.08)	2								
11	RE-TRIF - P4	20/03/2018 13:17	10.4	6	0	N/A (B.02.08)	N/A								
12	RE-TRIF - P5	21/03/2018 13:17	8.6	5	0.5	N/A (B.02.08)	N/A	1,745	1,989	0.8	9.9	3,859	4,297	0.5	5.3
13	RE-TRIF - P6	22/03/2018 13:17	333.7	208	4.2	N/A (B.02.08)	N/A	1,620	2,160	12.2	3.6	2,190	4,645	50.9	15.3



Interpretation: RNA integrities were assessed using the Agilent Bioanalyser 2100 which calculates RIN values of assayed RNAs (Table and Figure above).

The RNA integrity for buffy coat samples extracted by Trizol was above 7 for patient 6 only, indicating good quality total RNA.

The electropherograms for the total RNA present two distinct peaks (28S, and 18 S) in patient samples 3 and 6. Sample 3 has other peaks, indicating degradation.



<u>Interpretation:</u> (repeat attempt to assess the quality of the samples)

RNA integrities were assessed using the Agilent Bioanalyser 2100 which calculates RIN values of assayed RNAs (table and figure above).

The RNA integrity for buffy coat samples extracted by Trizol. None of the samples were above 7, indicating the absence of good quality total RNA.

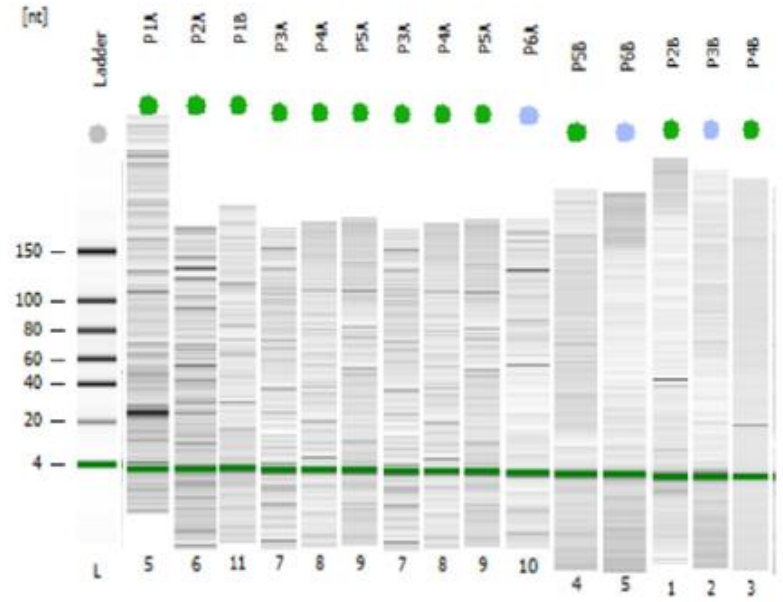
The electropherograms for the total RNA present no distinct peaks (28S, and 18 S) in patient samples. This may suggest poor quality samples with degradation.

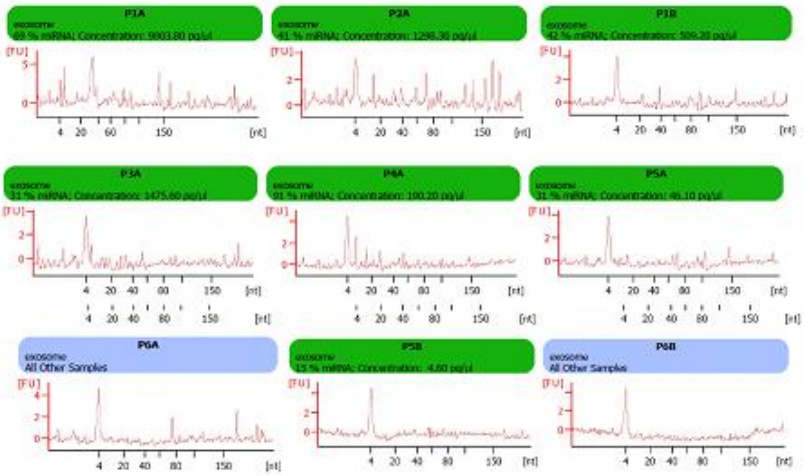
B. Small RNA

1. Agilent Bioanalyser

I. Serum Tubes (exosome)

		Agilent						Agilent reading: Region table for sample											
								small RNA						miRNA					
#	Sample ID	Date and time	small RNA Concentration [pg/µl]	miRNA Concentration [pg/µl]	miRNA / Small RNA Ratio [%]	Result Flagging Label		Name From [nt]	To [nt]	Average Size [nt]	Size distribution in CV [%]	Conc. [pg/µl]	% of Total Color	Name From [nt]	To [nt]	Average Size [nt]	Size distribution in CV [%]	Conc. [pg/µl]	% of Total Color
small RNA																			
Serum																			
1	Exosom - p1A	14/03/2018 11:41	14,226.80	9,803.80	69	69 % miRNA; Concentration: 9803.80 pg/µl	0	33	7	84	100	14,226.70	100	10	40	30	20.1	9,803.80	69
2	Exosom - p1B	15/03/2018 11:41	1,210.10	509.2	42	42 % miRNA; Concentration: 509.20 pg/µl	0	23	4	46	100	1,210.10	100	10	40	25	39.6	509.2	42
3	Exosom - p2A	14/03/2018 11:41	3,171.10	1,298.30	41	41 % miRNA; Concentration: 1298.30 pg/µl	0	21	1	58	100	3,171.10	100	10	40	24	39.1	1,298.30	41
4	Exosom - p2B	14/03/2018 12:18	955.1	266.1	28	28 % miRNA; Concentration: 266.10 pg/µl	0	23	7	60	100	955.1	100	10	40	20	39.8	266.1	28
5	Exosom - p3A	14/03/2018 11:41	4,755.80	1,475.60	31	31 % miRNA; Concentration: 1475.60 pg/µl	0	21	4	67	100	4,755.80	100	10	40	23	40	1,475.60	31
6	Exosom - p3B	14/03/2018 12:18	3.6	0	0	All Other Samples	0	22	4	4	19.3	3.6	100	10	40	0	0	0	0
7	Exosom - p4A	14/03/2018 11:41	209.9	190.2	91	91 % miRNA; Concentration: 190.20 pg/µl	0	22	1	10	100	209.9	100	10	40	16	46.9	190.2	91
8	Exosom - p4B	14/03/2018 12:18	1,167.70	1,103.50	94	94 % miRNA; Concentration: 1103.50 pg/µl	0	21	7	22	100	1,167.70	100	10	40	24	1	1,103.50	94
9	Exosom - p5A	14/03/2018 11:41	150	46.1	31	31 % miRNA; Concentration: 46.10 pg/µl	0	22	5	34	100	150	100	10	40	16	7.3	46.1	31
10	Exosom - p5B	14/03/2018 12:18	30.1	4.6	15	15 % miRNA; Concentration: 4.60 pg/µl	0	20	7	7	100	30.1	100	10	40	36	10	4.6	15
11	Exosom - p6A	14/03/2018 11:41	178	0	0	All Other Samples	0	22	9	56	100	178	100	10	40	0	0	0	0
12	Exosom - p6B	14/03/2018 12:18	19.2	0	0	All Other Samples	0	20	4	4	100	19.2	100	10	40	0	0	0	0



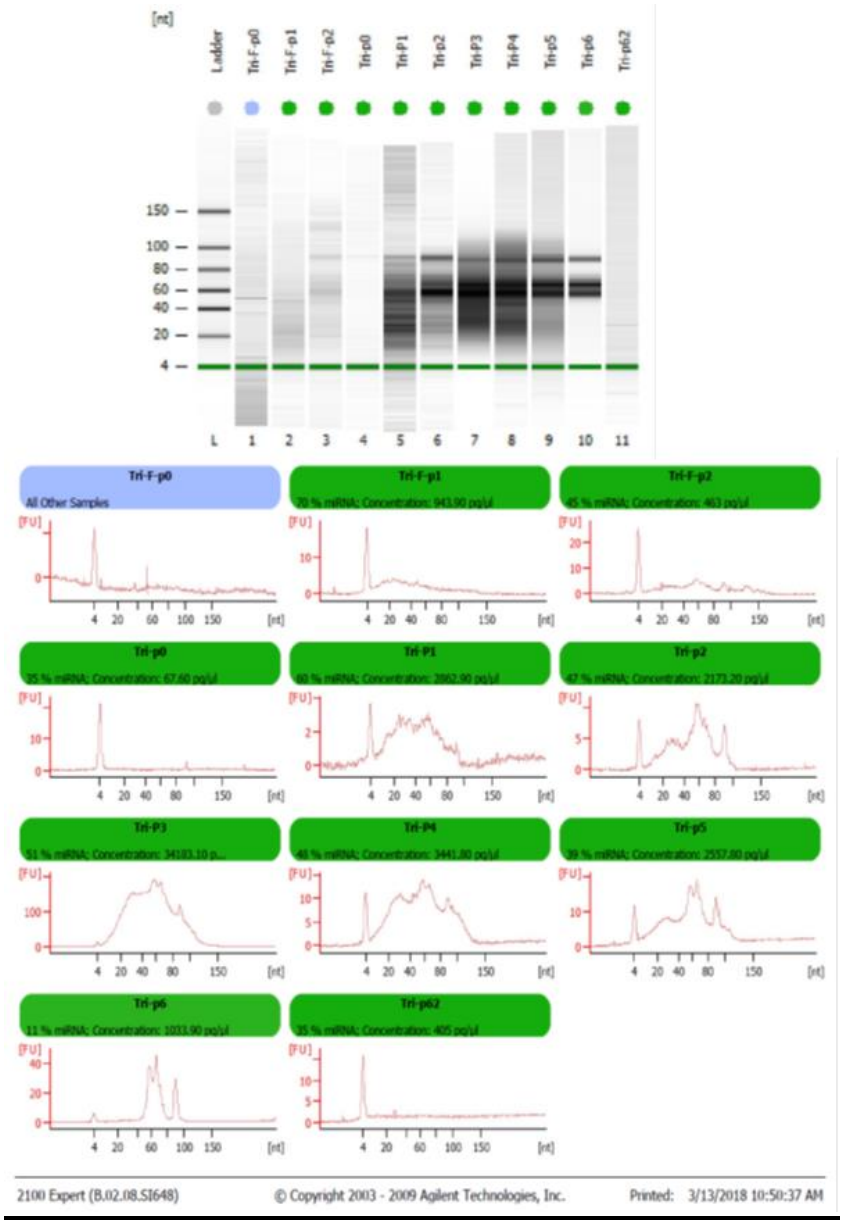


Interpretation:

Using SmallChip (Bio-SmallChip), data are shown for an exosome (the concentration in pg and percentage of small RNA and microRNA). It shows good quality.

II.Buffy Coat (EDTA) or lymphocytes

		Agilent					Agilent reading: Region table for sample											
							small RNA						miRNA					
#	Sample ID	Date and time	small RNA Concentration [pg/µl]	miRNA Concentration [pg/µl]	miRNA / Small RNA Ratio [%]	Result Flagging Label	Name From [nt]	To [nt]	Average Size [nt]	Size distribution in CV [%]	Conc. [pg/µl]	% of Total Color	Name From [nt]	To [nt]	Average Size [nt]	Size distribution in CV [%]	Conc. [pg/µl]	% of Total Color
small RNA																		
Buffy coat (PBMC or lymphocytes)																		
1	Trisol - p1	13/03/2018 10:08	4,750.80	2,862.90	60	60 % miRNA; Concentration: 2862.90 pg/µl	0	26	55	94.4	4,750.80	100	10	40	26	31.8	2,862.90	60
2	Trisol - p2	13/03/2018 10:08	4,634.10	2,173.20	47	47 % miRNA; Concentration: 2173.20 pg/µl	0	29	54	64.1	4,634.10	100	10	40	26	29.5	2,173.20	47
3	Trisol - p3	13/03/2018 10:08	67,593.70	34,183.10	51	51 % miRNA;Concentration: 34183.10 pg/µl	0	47	56	51.1	67,593.70	100	10	40	28	27.7	34,183.10	51
4	Trisol - p3-2nd	Not done																
5	Trisol - p4	13/03/2018 10:08	7,200.20	3,441.80	48	49 % miRNA; Concentration: 3441.80 pg/µl	1	52	63	71.6	7,200.20	100	11	40	26	30	3,441.80	48
6	Trisol - p5	13/03/2018 10:08	6,582.90	2,557.80	39	39 % miRNA; Concentration: 2557.80 pg/µl	0	257	75	78.7	6,582.90	100	10	40	26	32.1	2,557.80	39
7	Trisol - p6	13/03/2018 10:08	9,535.80	1,033.90	11	11 % miRNA; Concentration: 1033.90 pg/µl	0	259	75	57.4	9,535.80	100	10	40	26	33.1	1,033.90	11

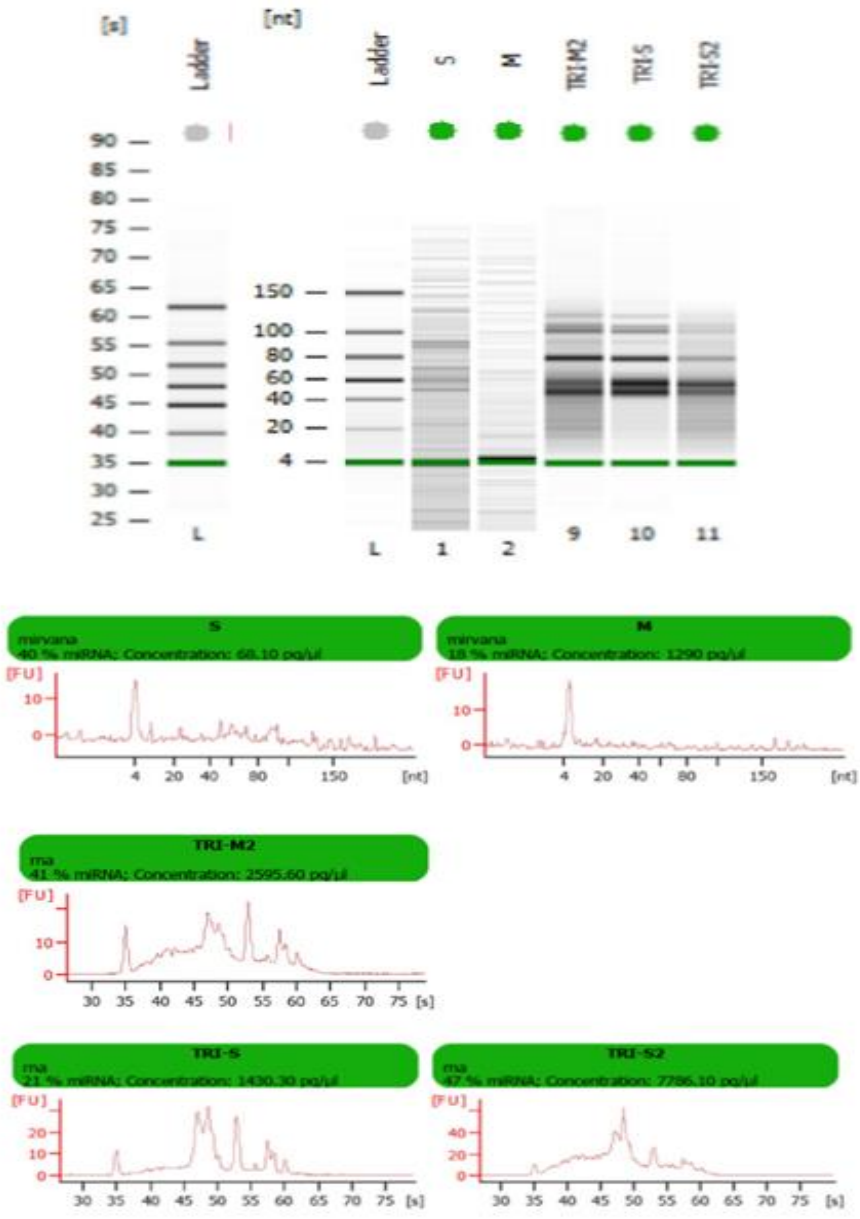


Interpretation:

Using SmallChip (Bio-SmallChip), data are shown for the buffy coat samples (the concentration in pg and percentage of small RNA and microRNA). It shows good quality.

III. Blood samples analysis (results for an experiment to evaluate best extraction kit for miRNA (MirVana vs. Trizol) from buffy coat

		Agilent						Agilent reading: Region table for sample											
#	Sample ID	Date and time	small RNA Concentration [pg/µl]	miRNA Concentration [pg/µl]	miRNA / Small RNA Ratio [%]	Result Flagging Label		small RNA						miRNA					
							Name From [nt]	To [nt]	Average Size [nt]	Size distribution in CV [%]	Conc. [pg/µl]	% of Total Color		Name From [nt]	To [nt]	Average Size [nt]	Size distribution in CV [%]	Conc. [pg/µl]	% of Total Color
small RNA																			
Buffy coat (PBMC or lymphocytes) Experimental on placebo healthy samples																			
1	Patient M Mirvana Extr	14/03/2018 11:41	7,078.50	1,290.00	18	18 % miRNA; Concentration: 1290 pg/µl	0	20	14	100	7,078.50	100		10	40	19	46	1,290.00	18
2	Patient S Mirvana Extr	14/03/2018 11:41	170.6	68.1	40	40 % miRNA; Concentration: 68.10 pg/µl	0	20	22	100	170.6	100		10	40	16	40.3	68.1	40
3	Patient M Trizol Extr		6,275.60	2,595.60	41	41 % miRNA; Concentration: 2595.60 pg/µl	0	28	66	61.7	6,275.60	100		10	40	26	31.6	2,595.60	41
4	Patient S Trizol Extr		16,694.50	7,786.10	47	47 % miRNA; Concentration: 7786.10 pg/µl	0	29	60	59.1	16,694.50	100		10	40	26	32.4	7,786.10	47
5	Patient S Trizol Extr (mag beads cells isol.)		6,747.00	1,430.30	21	21 % miRNA; Concentration: 1430.30 pg/µl	0	28	68	46.2	6,747.00	100		10	40	27	29.9	1,430.30	21



Interpretation:

Using SmallChip (Bio-SmallChip), data are shown for an experiment comparing mirVana and Trisol extraction kits for buffy coat (the concentration in pg and percentage of small RNA and microRNA). It shows good quality.

Summary of the results:

Samples collected at baseline when the patients had not yet received IGIV. Extraction from different blood products was used to determine the best resource for the study, including whole blood (PAX tube), serum exosome and buffy coats. Multiple trials on control samples were also used to evaluate the best purification method with better yields including the use of Trizol and mirVana enrichment methods. Preliminary results from the six patients and from the different sources are promising. We managed to extract good quality RNA and small RNA from most of the samples. We noticed that using the Nanodrop for the readings was misleading and yielded some undependable results, while the Agilent analyser gave more consistent readings. We plan to primarily read all samples using the Agilent analyser. A few technical `issues are limitations, which will be corrected with the subsequent sample extractions. No microbiomes were used, so we currently have no data because the extraction kits had not been received when writing this report. The subsequent parts were done afterwards, when the extraction kits and arrays had arrived; the next steps were done when patients were on IGIV and multivitamins (interventional stage).

Extraction from PAX tubes:

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type
1	Pax_Maryam overnight	LAB	17/10/2018 03:14:05 م	53.8	ng/µl	1.345	0.649	2.07	1.55	RNA
2	Pax_Amal Fresh	LAB	17/10/2018 03:15:50 م	65.7	ng/µl	1.642	0.813	2.02	1.88	RNA
3	Pax_Amal 4 hrs	LAB	17/10/2018 03:17:01 م	46.6	ng/µl	1.165	0.655	1.78	-1.46	RNA
4	Pax_R_Maryam overnight	LAB	17/10/2018 03:18:13 م	6.7	ng/µl	0.167	0.074	2.25	0.53	RNA
5	Pax_R_Amal Fresh	LAB	17/10/2018 03:19:07 م	0.6	ng/µl	0.015	0.017	0.85	-0.01	RNA
6	Pax_R_Amal 4 hrs	LAB	17/10/2018 03:19:58 م	17.3	ng/µl	0.434	0.265	1.64	-0.31	RNA

2) Trials to assess best method for the extraction of miRNA from serum exosome and microbiome DNA from stool:

#	Sample ID	Date and Time	Nucleic Acid Conc. ng/µl	A260	A280	260/280	260/230	Sample Type	Factor
1	Repeat Nano reading Exp 1: microKit No DNase	12/11/2018 08:01:16 ص	21.4	0.535	0.351	1.52	0.1	RNA	40
2	Repeat Nano reading Exp 1: microKit No DNase rem	12/11/2018 08:02:47 ص	18.1	0.451	0.292	1.54	0.15	RNA	40
3	Repeat Nano reading Exp 1: microKit DNase	12/11/2018 08:03:42 ص	9.2	0.23	0.141	1.63	0.11	RNA	40
4	Repeat Nano reading Exp 1: microKit DNase rem	12/11/2018 08:04:20 ص	6.9	0.173	0.099	1.74	0.51	RNA	40
5	Repeat Nano reading Exp 2: microKit with homog	12/11/2018 08:05:41 ص	17.6	0.44	0.299	1.48	0.67	RNA	40
6	Repeat Nano reading Exp 2: microKit no homo/ no Qiazol	12/11/2018 08:06:37 ص	15.4	0.386	0.222	1.74	0.03	RNA	40
7	Repeat Nano reading Exp 3: microKit and serumkit: sample 1 il column	12/11/2018 08:08:16 ص	76.2	1.906	1.314	1.45	0.19	RNA	40
8	Repeat Nano reading Exp 3: microKit and serumkit: sample 2 il column	12/11/2018 08:08:56 ص	39.1	0.977	0.684	1.43	0.33	RNA	40
9	Repeat Nano reading Exp 3: microKit and serumkit: sample 3 j column	12/11/2018 08:09:28 ص	30.5	0.763	0.515	1.48	0.16	RNA	40
10	Repeat Nano reading Exp 3: microKit and serumkit: sample 4 j column	12/11/2018 08:10:00 ص	28.1	0.703	0.484	1.45	0.83	RNA	40
11	Repeat Nano reading Exp 3: microKit and serumkit: sample 5 micro column	12/11/2018 08:10:34 ص	48.8	1.219	0.72	1.69	0.21	RNA	40
12	Repeat Nano reading Exp 3: microKit and serumkit: sample 8 micro column	12/11/2018 08:11:03 ص	63.8	1.596	1.084	1.47	0.17	RNA	40
13	Repeat Nano reading Exp 3: microKit and serumkit: sample 9 micro column	12/11/2018 08:11:33 ص	46.5	1.162	0.797	1.46	0.13	RNA	40
14	Repeat Nano reading Exp 4: invitrokit conc sample 1	12/11/2018 08:16:08 ص	7.8	0.194	0.106	1.83	0.04	RNA	40
15	Repeat Nano reading Exp 4: invitrokit conc sample 1 rem in gel	12/11/2018 08:16:45 ص	18.7	0.469	0.055	8.56	0.24	RNA	40
16	Repeat Nano reading Exp 4: invitrokit conc sample 1 rem in column	12/11/2018 08:18:33 ص	3.7	0.091	0.076	1.21	0.28	RNA	40
17	Repeat Nano reading Exp 4: invitrokit dil sample 2	12/11/2018 08:19:59 ص	2.5	0.063	0.022	2.92	0.03	RNA	40
18	Repeat Nano reading Exp 4: invitrokit dil sample 2 rem in column	12/11/2018 08:21:09 ص	0.4	0.011	0.023	0.48	0.12	RNA	40
19	Repeat Nano reading Exp 4: invitrokit con sample 1 repeat nano	12/11/2018 08:22:12 ص	6.7	0.168	0.089	1.87	0.04	RNA	40

Control trial to assess the best method for the extraction of miRNA from exosomes

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1st reading											
1	sample 1 POWER WITH OMNI	LAB	17/07/2018 02:56:32 م	25.6	ng/µl	0.513	0.302	1.7	1.13	DNA	50
2	sample 2 POWER STOOL DIRECT	LAB	17/07/2018 02:58:03 م	66.3	ng/µl	1.326	0.764	1.74	0.99	DNA	50
3	sample 3 FAST OMNI	LAB	17/07/2018 03:01:35 م	74.1	ng/µl	1.482	0.791	1.87	1.23	DNA	50
4	sample 4 FAST STOOL DIRECT	LAB	17/07/2018 03:02:43 م	79.3	ng/µl	1.586	0.813	1.95	1.25	DNA	50
2nd reading											
1	sample 1 conrtol powerfecal omni	LAB	13/11/2018 11:58:34 ص	43.4	ng/µl	0.868	0.483	1.8	1.6	DNA	50
2	sample 1 conrtol powerfecal omni	LAB	13/11/2018 11:59:36 ص	21.2	ng/µl	0.424	0.267	1.59	1.82	DNA	50
3	sample 1 conrtol powerfecal omni	LAB	13/11/2018 12:00:04 م	23	ng/µl	0.46	0.324	1.42	1.63	DNA	50
4	sample 2 conrtol powerfecal stool direct	LAB	13/11/2018 12:00:51 م	27.9	ng/µl	0.557	0.251	2.22	1.04	DNA	50
5	sample 2 conrtol powerfecal stool direct	LAB	13/11/2018 12:01:22 م	47.7	ng/µl	0.955	0.529	1.8	1.52	DNA	50
6	sample 3 Fast kit omni	LAB	13/11/2018 12:06:22 م	67.6	ng/µl	1.353	0.727	1.86	1.29	DNA	50
7	sample 4 Fast kit stool direct	LAB	13/11/2018 12:07:17 م	63.6	ng/µl	1.272	0.655	1.94	1.62	DNA	50
after clean up											
5	sample 1 PF OMNI	LAB	13/11/2018 02:55:48 م	62.9	ng/µl	1.258	0.705	1.78	1.5	DNA	50
6	sample 2 PF stool	LAB	13/11/2018 02:56:51 م	60.9	ng/µl	1.217	0.662	1.84	1.85	DNA	50
7	sample 3 fast OMNI	LAB	13/11/2018 02:57:42 م	452.2	ng/µl	9.043	5.047	1.79	1.34	DNA	50
8	sample 3 fast OMNI	LAB	13/11/2018 02:58:29 م	461.7	ng/µl	9.233	5.137	1.8	1.34	DNA	50
9	sample 4 fast OMNI	LAB	13/11/2018 02:59:12 م	415.9	ng/µl	8.319	4.491	1.85	1.62	DNA	50
11	sample 4 fast stool	LAB	13/11/2018 03:00:34 م	414.7	ng/µl	8.293	4.471	1.85	1.63	DNA	50

Microbiome DNA extraction Nanodrop readings before and after the ethanol clean- up step of control samples

APPENDIX VI- Microbiome array methodology

Microbiome array:

Please refer to manufacturer's instructions for further details:

https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets/LSG/manuals/703335_Axiom_24F_ManualWrkflw_UG.pdf

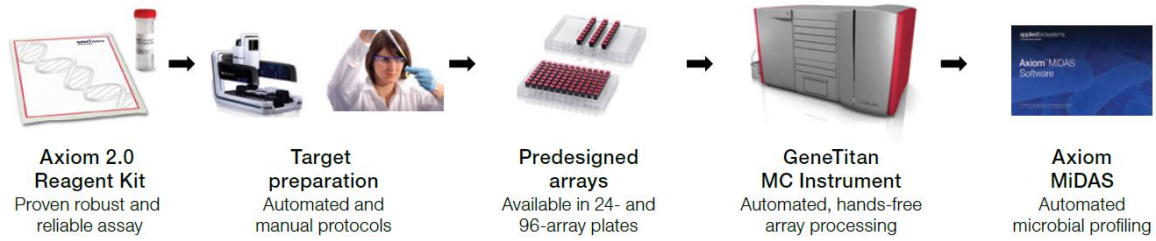


Figure 1. The Axiom Microbiome Solution workflow. From cDNA or genomic DNA to easy-to-use Axiom Microbial Detection Analysis Software (MiDAS), the Axiom Microbiome Solution provides the complete answer for microbial profiling.

Table 1. Target categories represented on the Axiom Microbiome Array.

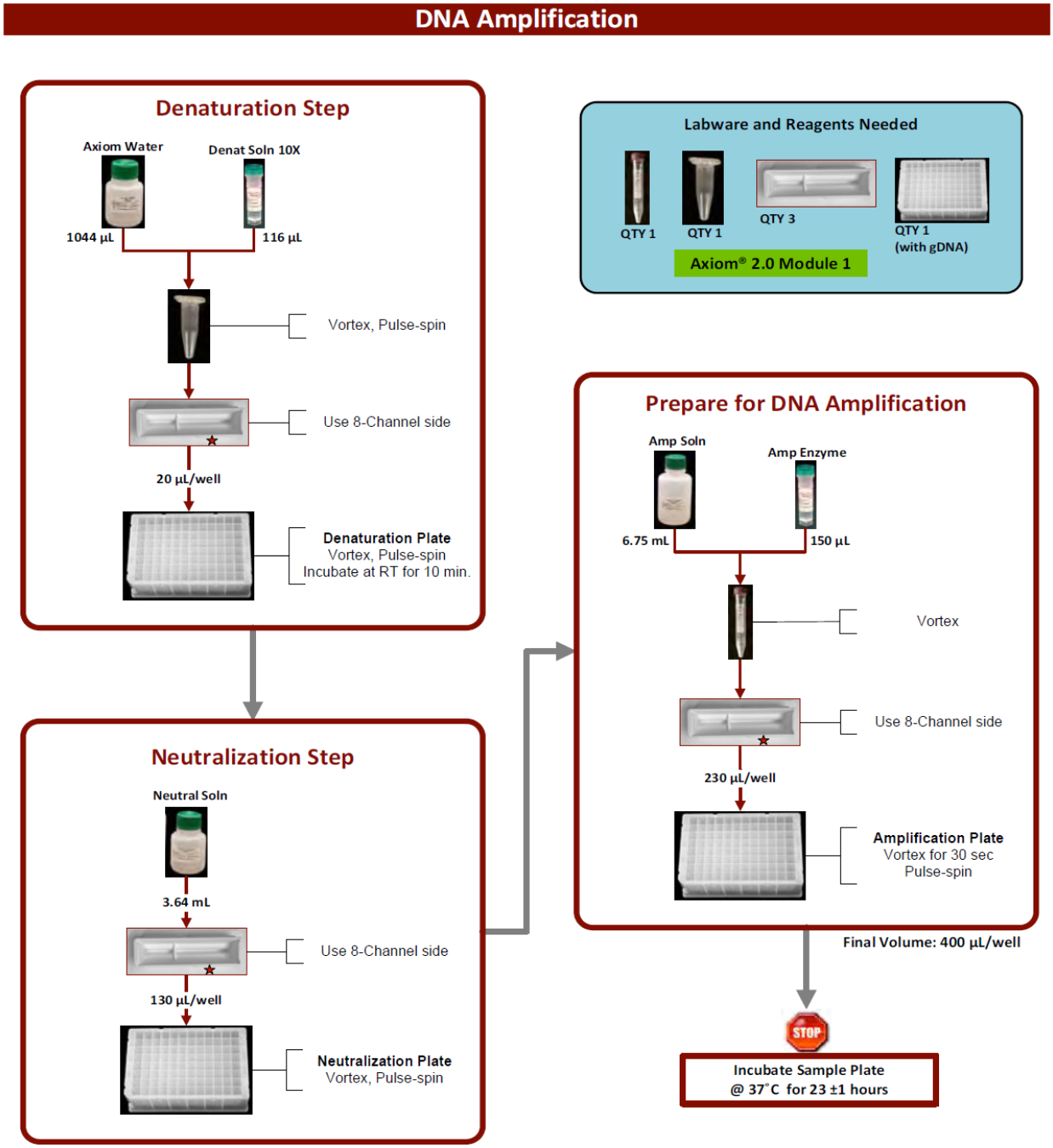
Category	Number of families*	Number of species	Target sequences**
Archaea	31	370	606
Bacteria	278	6,901	34,254
Fungi	121	381	658
Protozoa	30	91	229
Viral	100	4,770	99,808
Total	**560**	**12,513**	**135,555**

* Number of families reflects NCBI known family classifications as of October 2014. Unknown or ambiguous family-associated targets are not included in the total number of families count, but may be included with "unclassified" or "unknown" family assignments in software results and output files.

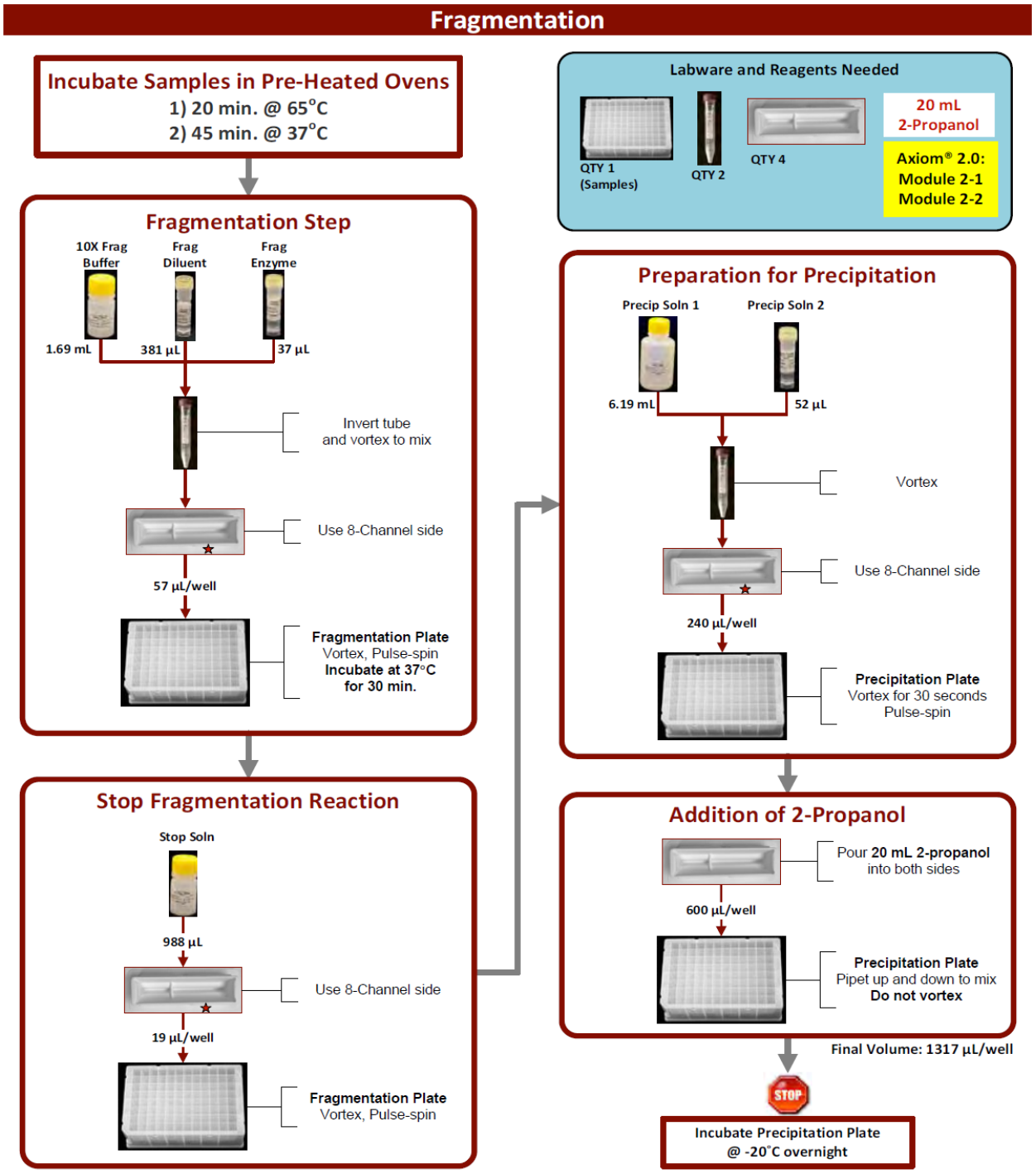
** Multiple probes have been designed to interrogate each target sequence. A probe may be common in more than one organism (family-conserved) or unique to a particular strain (target-specific).

The protocol for manual target preparation composed of

Stage 1: DNA amplification

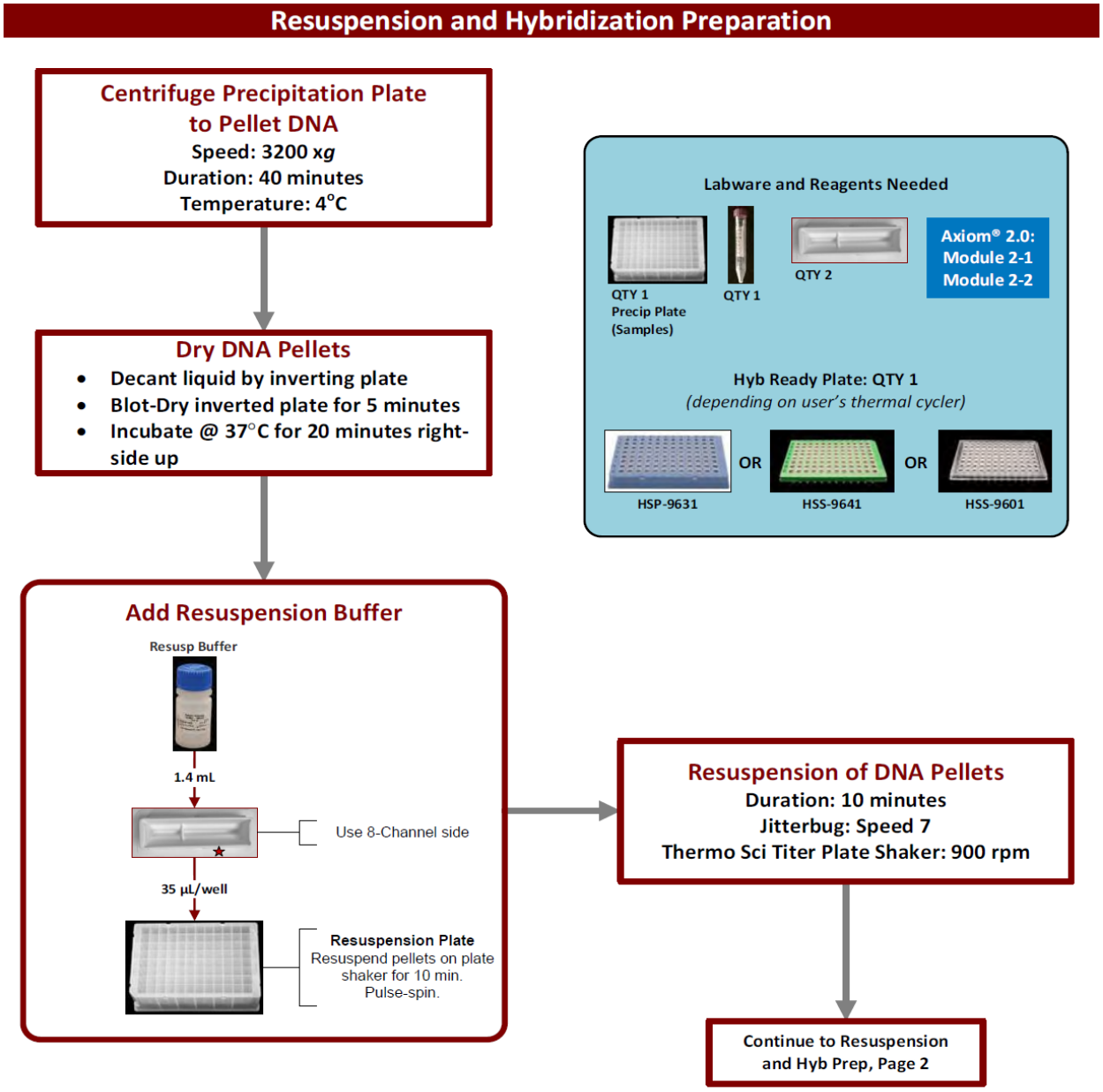


Stage 2: Fragmentation and precipitation

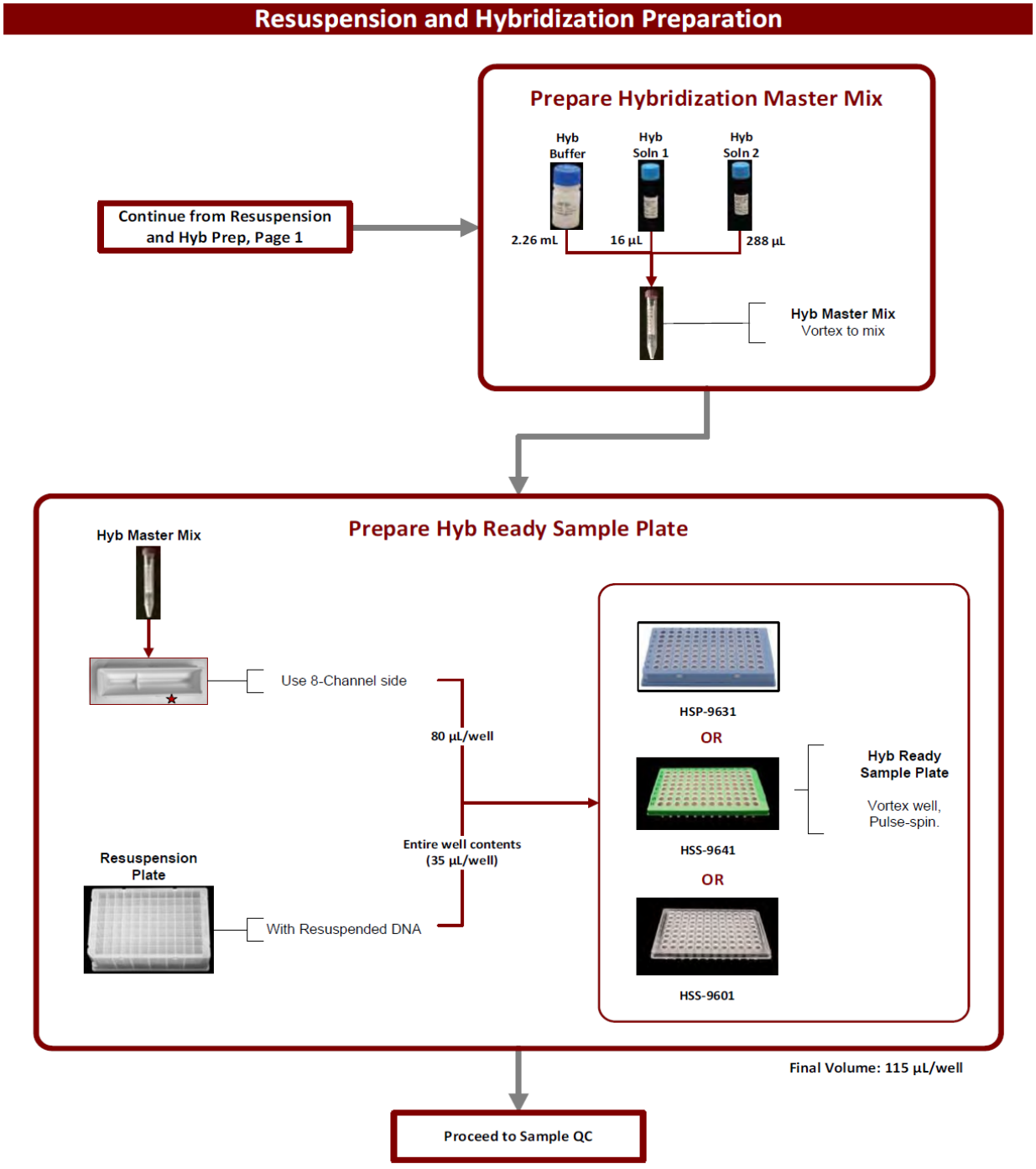


Stage 3: Centrifuge and drying, re-suspension and hybridisation preparation & QC

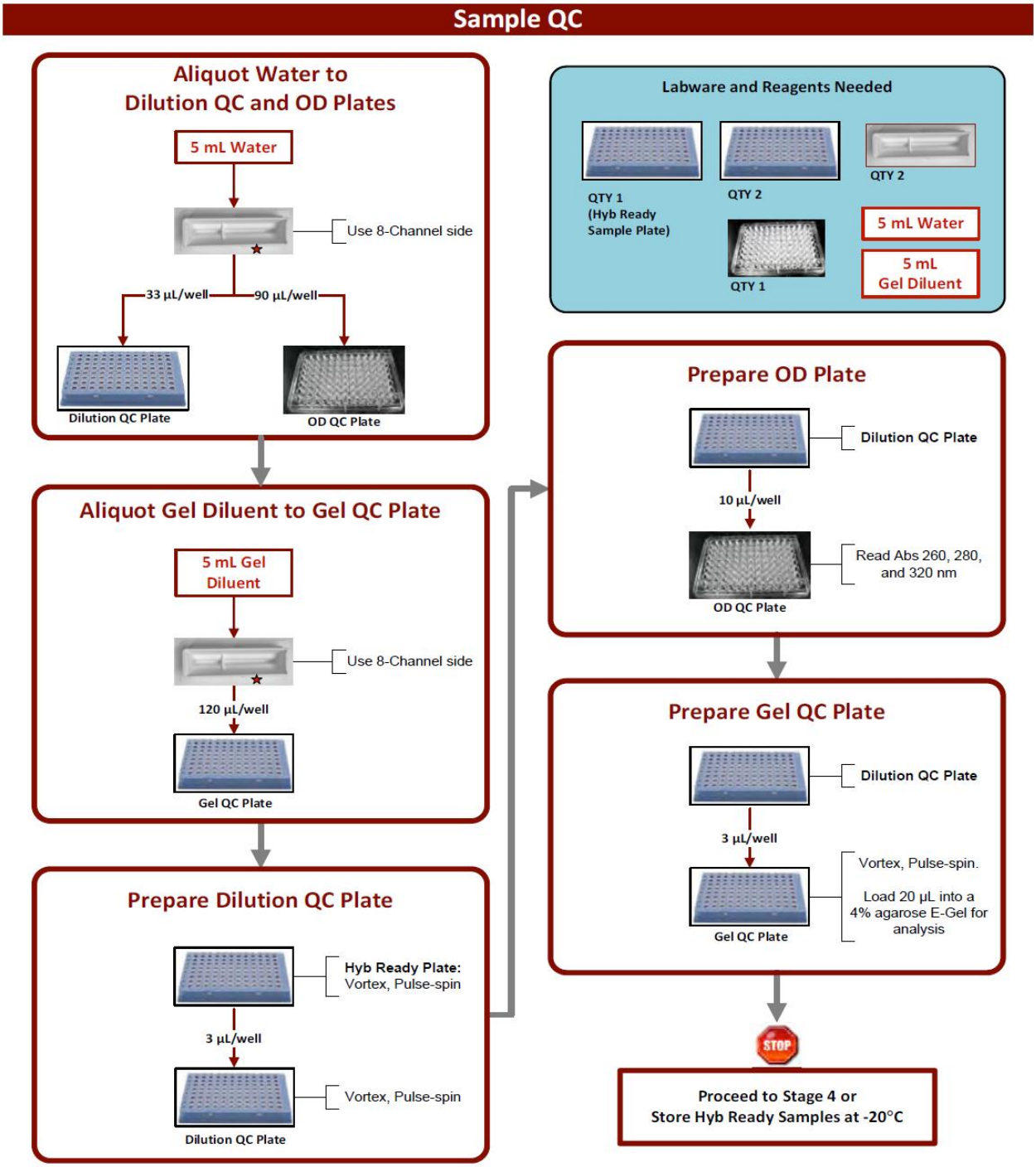
Stage 3A: Centrifuge precipitation plate and dry the DNA pellet



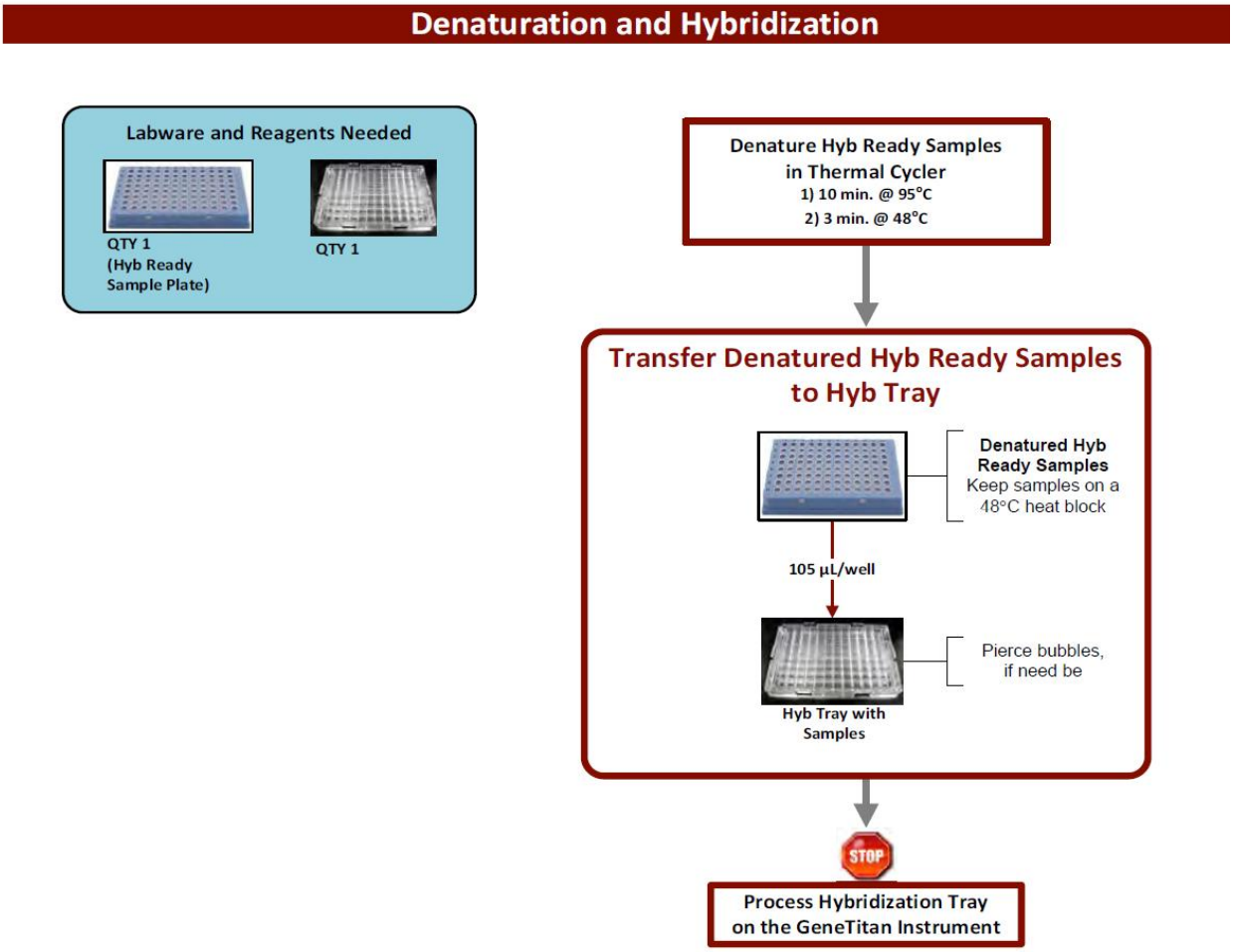
Stage 3B: Resuspension and hybridisation preparation



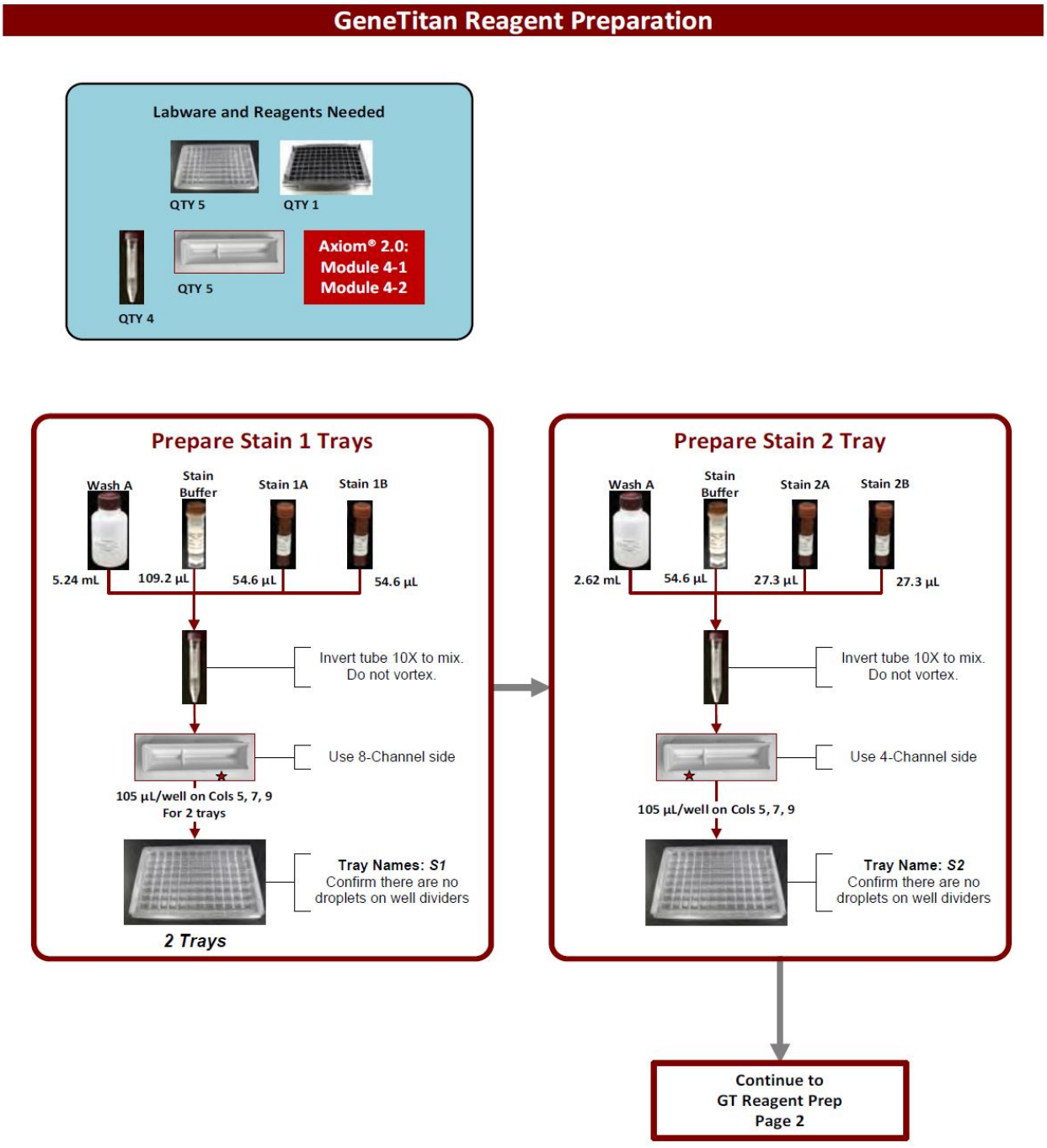
Stage 3C: Perform quantitation and fragmentation QC checks (recommended)



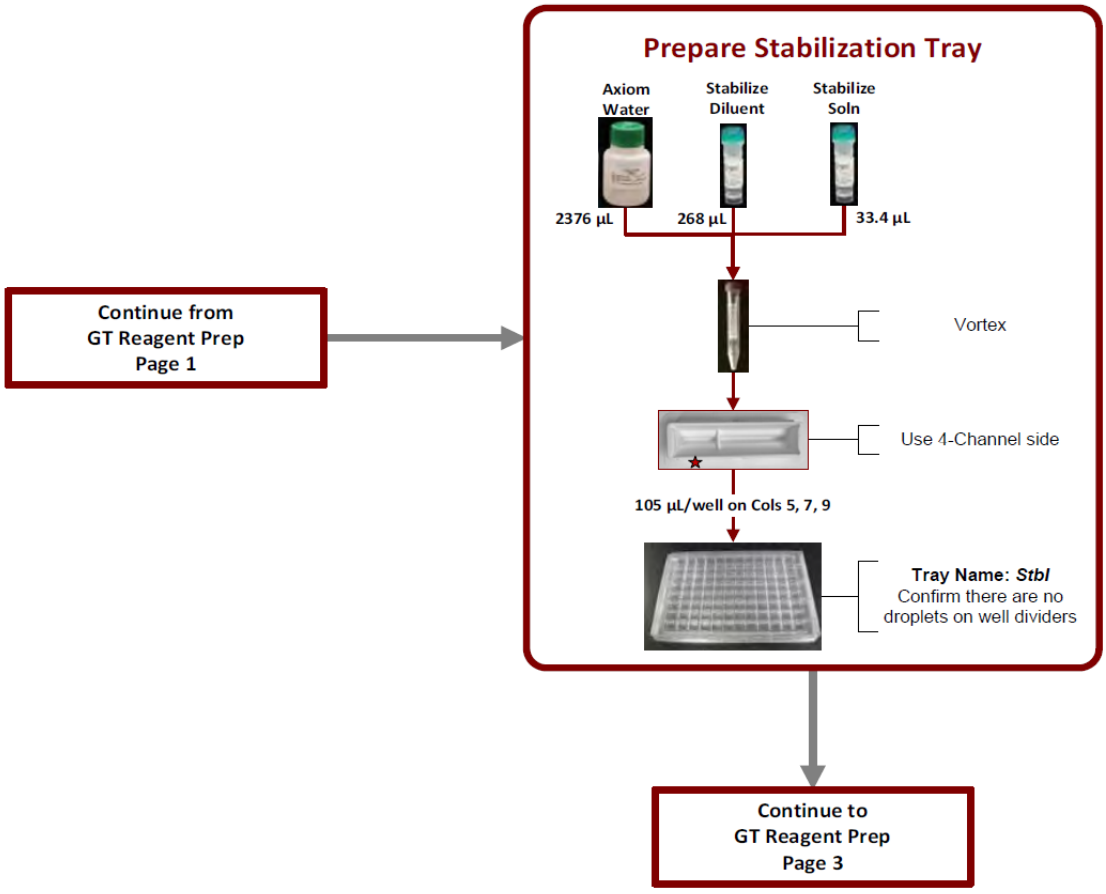
Stage 4: Denaturation and hybridisation



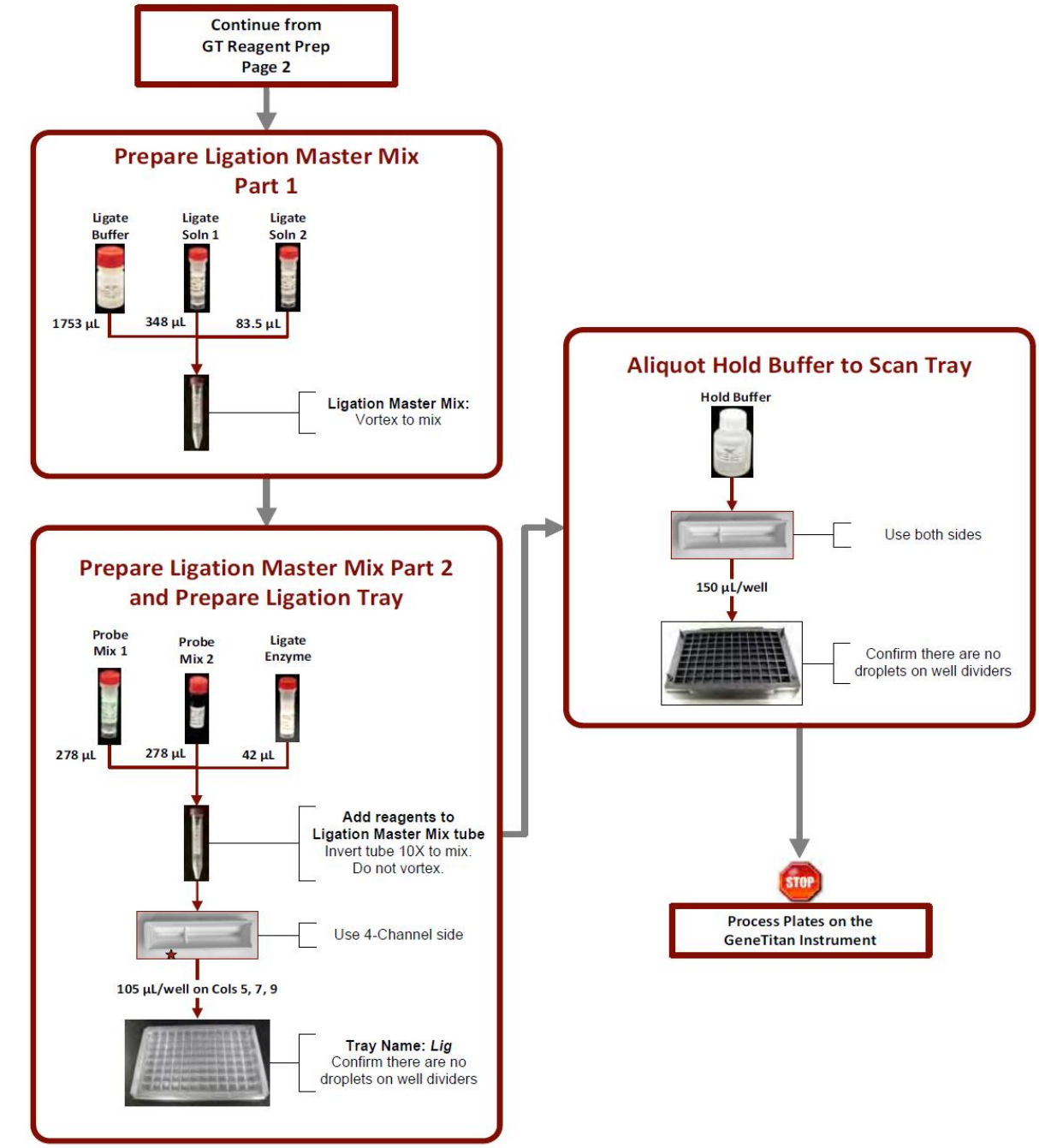
Stage 5: GeneTitan™ reagent preparation



GeneTitan Reagent Preparation



GeneTitan Reagent Preparation



APPENDIX VII- Certificates of training

GULF SCIENTIFIC CORPORATION

Certificate of Attendance

This is to certify that

DR. MARIAM AL-NESF

Has successfully attended and completed

HANDS ON GENECHIP™ MIRNA 4.1 ARRAY PLATE (THERMO) AND BASIC TAC SOFTWARE ANALYSIS

Venue:
ANTI-DOPING LAB, QATAR.

Date:
DEC. 13-15, 2018

MUAD ABU TAYEH
Trainer

P.O. Box 17010 JEBEL ALI FREE ZONE DUBAI-U.A.E. GULF SCIENTIFIC CORPORATION

applied biosystems
Authorised Distributor

GULF SCIENTIFIC CORPORATION

Certificate of Attendance

This is to certify that

DR. MARIAM AL-NESF

Has successfully attended and completed

HANDS ON TRAINING FOR AXIOM™ MICROBIOME, 24 ARRAY PLATE (THERMO) AND BASIC AXIOM MIDAS SOFTWARE ANALYSIS

Venue:
ANTI-DOPING LAB, QATAR.

Date:
NOV. 25-29, 2018

MUAD ABU TAYEH
Trainer

P.O. Box 17010 JEBEL ALI FREE ZONE DUBAI-U.A.E. GULF SCIENTIFIC CORPORATION

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APPENDIX VIII- Microbiome DNA assessment using the nanodrop

Nanodrop quantification after extraction

Microbiome DNA assessment using the Nanodrop (section 4.5.3.2.)

#	Sample ID	Nucleic Acid Conc. ng/µl	A260	A280	260/280	260/230	Sample Type	Factor	Date and Time
1	microbiome P09 PQ-post heat @37 degree	8.5	0.169	0.152	1.12	1.1	DNA	50	20/11/2018 04:13:43 م
2	microbiome P10 PQ-post heat @37 degree	57.7	1.153	0.689	1.67	1.67	DNA	50	20/11/2018 04:14:35 م
3	microbiome P11 PQ-post heat @37 degree	109.4	2.187	1.174	1.86	0.87	DNA	50	20/11/2018 04:15:24 م
4	microbiome P11 PQ-post heat @37 degree	44.3	0.885	0.526	1.68	1.85	DNA	50	20/11/2018 04:16:15 م
5	microbiome P12 PQ-post heat @37 degree	13.3	0.266	0.171	1.55	2.39	DNA	50	20/11/2018 04:17:10 م
6	microbiome P12 PQ-post heat @37 degree	15.4	0.308	0.199	1.55	1.9	DNA	50	20/11/2018 04:17:59 م
7	microbiome P13 PQ-post heat @37 degree	14.9	0.297	0.2	1.49	1.79	DNA	50	20/11/2018 04:18:59 م
8	microbiome P13 PQ-post heat @37 degree	12.6	0.253	0.171	1.48	2.35	DNA	50	20/11/2018 04:19:29 م
9	microbiome P14 PQ-post heat @37 degree	68.1	1.362	0.773	1.76	1.92	DNA	50	20/11/2018 04:20:14 م
10	microbiome P15 PQ-post heat @37 degree	21.8	0.435	0.297	1.46	1.72	DNA	50	20/11/2018 04:20:54 م
11	microbiome P15 PQ-post heat @37 degree	19.6	0.392	0.255	1.54	1.86	DNA	50	20/11/2018 04:21:34 م
12	microbiome P16 PQ-post heat @37 degree	40	0.801	0.461	1.74	2.03	DNA	50	20/11/2018 04:22:17 م
13	microbiome P17 PQ-post heat @37 degree	36	0.72	-0.007	-108.99	1.83	DNA	50	20/11/2018 04:23:07 م
14	microbiome P17 PQ-post heat @37 degree	17	0.34	0.204	1.67	4.02	DNA	50	20/11/2018 04:23:49 م
15	microbiome P18 PQ-post heat @37 degree	30.9	0.618	0.357	1.73	1.49	DNA	50	20/11/2018 04:24:35 م
16	microbiome P19 PQ-post heat @37 degree	77	1.539	0.849	1.81	1.76	DNA	50	20/11/2018 04:25:32 م
17	microbiome P20 PQ-post heat @37 degree	70.7	1.413	0.783	1.8	1.9	DNA	50	20/11/2018 04:26:47 م
18	microbiome P21 PQ-post heat @37 degree	138.9	2.779	1.611	1.72	1	DNA	50	20/11/2018 04:27:37 م
19	microbiome P21 PQ-post heat @37 degree	29.8	0.595	0.342	1.74	2.69	DNA	50	20/11/2018 04:28:08 م
20	microbiome P22 PQ-post heat @37 degree	7.1	0.143	0.083	1.73	16.53	DNA	50	20/11/2018 04:29:16 م
21	microbiome P22 PQ-post heat @37 degree	8	0.159	0.092	1.74	6.86	DNA	50	20/11/2018 04:29:56 م
22	microbiome P23 PQ-post heat @37 degree	9.5	0.191	0.1	1.91	-21.29	DNA	50	20/11/2018 04:30:54 م
23	microbiome P23 PQ-post heat @37 degree	116.5	2.33	1.198	1.95	0.6	DNA	50	20/11/2018 04:31:28 م
24	microbiome P23 PQ-post heat @37 degree	50.5	1.011	0.304	3.32	0.9	DNA	50	20/11/2018 04:31:56 م
25	microbiome P23 PQ-post heat @37 degree	9.1	0.182	0.096	1.9	-4.4	DNA	50	20/11/2018 04:32:33 م
26	microbiome P24 PQ-post heat @37 degree	41.5	0.831	0.463	1.79	2.57	DNA	50	20/11/2018 04:33:23 م
27	microbiome P25 PQ-post heat @37 degree	51.5	1.029	0.572	1.8	1.8	DNA	50	20/11/2018 04:34:21 م
28	microbiome P26 PQ-post heat @37 degree	54.3	1.086	0.635	1.71	1.6	DNA	50	20/11/2018 04:35:12 م
29	microbiome P27 PQ-post heat @37 degree	35.7	0.715	0.434	1.65	1.07	DNA	50	20/11/2018 04:36:02 م
30	microbiome P27 PQ-post heat @37 degree	8	0.159	0.106	1.51	2.01	DNA	50	20/11/2018 04:36:42 م
31	microbiome P27 PQ-post heat @37 degree	9.3	0.186	0.112	1.67	2.13	DNA	50	20/11/2018 04:37:19 م
32	microbiome P28 PQ-post heat @37 degree	38.3	0.765	0.424	1.81	2.1	DNA	50	20/11/2018 04:38:41 م
33	microbiome P29 PQ-post heat @37 degree	113.7	2.274	1.222	1.86	2.09	DNA	50	20/11/2018 04:39:30 م
34	microbiome P29 PQ-post heat @37 degree	55.4	1.108	0.603	1.84	2.47	DNA	50	20/11/2018 04:40:13 م
35	microbiome P30 PQ-post heat @37 degree	36.1	0.721	0.401	1.8	2.91	DNA	50	20/11/2018 04:41:11 م
36	microbiome P32 PQ-post heat @37 degree	88.1	1.762	0.949	1.86	2.37	DNA	50	20/11/2018 04:42:26 م
37	microbiome P33 PQ-post heat @37 degree	22.9	0.457	0.261	1.75	3	DNA	50	20/11/2018 04:43:01 م
38	microbiome P33 PQ-post heat @37 degree	69.2	1.385	0.267	5.18	1.42	DNA	50	20/11/2018 04:43:27 م
39	microbiome P33 PQ-post heat @37 degree	22.4	0.448	0.253	1.78	3.44	DNA	50	20/11/2018 04:44:30 م
40	microbiome P34 PQ-post heat @37 degree	66.1	1.322	0.458	2.89	0.45	DNA	50	20/11/2018 04:45:16 م
41	microbiome P34 PQ-post heat @37 degree	-0.4	-0.008	0.017	-0.45	0.17	DNA	50	20/11/2018 04:45:45 م
42	microbiome P34 PQ-post heat @37 degree	-0.3	-0.006	0	-25.15	0.07	DNA	50	20/11/2018 04:46:36 م
43	microbiome P27 PQ-post heat @37 degree	8.7	0.174	0.096	1.81	1.8	DNA	50	20/11/2018 04:47:15 م
44	microbiome P34 PQ-post heat @37 degree	-0.7	-0.014	-0.015	0.95	0.16	DNA	50	20/11/2018 04:48:25 م
45	microbiome P34 PQ-post heat @37 degree	-0.5	-0.009	-0.003	2.98	0.1	DNA	50	20/11/2018 04:48:57 م
46	microbiome P34 PQ-post heat @37 degree	-0.5	-0.011	-0.017	0.63	0.1	DNA	50	20/11/2018 04:49:52 م
47	microbiome P34 PQ-post heat @37 degree	66.3	1.326	0.492	2.69	0.44	DNA	50	20/11/2018 04:53:21 م

Nanodrop readings after extraction, cleaning with ethanol and heating @ 37ºC for 37 samples of microbiome DNA.

APPENDIX IX- Quantification of samples post amplification and defragmentation.

Nanodrop quantification post-amplification and defragmentation

Quantification of samples post amplification and defragmentation and before the denaturation and hybridisation step (section 4.5.3.4.)

#	Sample ID	Nucleic Acid Conc. ng/µl	A260	A280	260/280	260/230	Sample Type	Factor	Date and Time
1	post amp & defrag blank	-0.4	-0.008	-0.017	0.48	0.51	DNA	50	27/11/2018 11:39:08 ص
2	post amp & defrag 1	125.5	2.51	1.341	1.87	1.72	DNA	50	27/11/2018 11:40:03 ص
3	post amp & defrag 2	101.3	2.026	1.09	1.86	1.67	DNA	50	27/11/2018 11:40:42 ص
4	post amp & defrag 3	108.5	2.17	1.176	1.85	1.69	DNA	50	27/11/2018 11:41:08 ص
5	post amp & defrag 4	106.4	2.128	1.142	1.86	1.7	DNA	50	27/11/2018 11:41:35 ص
6	post amp & defrag 5	106.8	2.137	1.145	1.87	1.72	DNA	50	27/11/2018 11:42:02 ص
7	post amp & defrag 6	106.1	2.121	1.135	1.87	1.71	DNA	50	27/11/2018 11:42:32 ص
8	post amp & defrag 7	108.7	2.173	1.174	1.85	1.71	DNA	50	27/11/2018 11:43:02 ص
9	post amp & defrag 8	105.5	2.11	1.127	1.87	1.69	DNA	50	27/11/2018 11:43:30 ص
10	post amp & defrag 9	108	2.16	1.176	1.84	1.71	DNA	50	27/11/2018 11:44:10 ص
11	post amp & defrag 10	107.4	2.148	1.17	1.84	1.7	DNA	50	27/11/2018 11:44:35 ص
12	post amp & defrag 11	106.8	2.135	1.152	1.85	1.7	DNA	50	27/11/2018 11:44:57 ص
13	post amp & defrag 12	105.9	2.118	1.145	1.85	1.71	DNA	50	27/11/2018 11:45:17 ص
14	post amp & defrag 13	106.4	2.127	1.151	1.85	1.68	DNA	50	27/11/2018 11:45:38 ص
15	post amp & defrag 14	108	2.159	1.169	1.85	1.7	DNA	50	27/11/2018 11:46:04 ص
16	post amp & defrag 15	109.6	2.191	1.187	1.85	1.71	DNA	50	27/11/2018 11:46:25 ص
17	post amp & defrag 16	117.4	2.347	1.269	1.85	1.72	DNA	50	27/11/2018 11:46:46 ص
18	post amp & defrag 17	105.1	2.103	1.141	1.84	1.67	DNA	50	27/11/2018 11:47:21 ص
19	post amp & defrag 18	106.5	2.131	1.155	1.85	1.7	DNA	50	27/11/2018 11:47:38 ص
20	post amp & defrag 19	105.6	2.112	1.153	1.83	1.69	DNA	50	27/11/2018 11:47:59 ص
21	post amp & defrag 20	113.6	2.273	1.222	1.86	1.7	DNA	50	27/11/2018 11:48:21 ص
22	post amp & defrag 21	109.7	2.194	1.185	1.85	1.69	DNA	50	27/11/2018 11:48:40 ص
23	post amp & defrag 22	108.7	2.175	1.178	1.85	1.68	DNA	50	27/11/2018 11:49:00 ص
24	post amp & defrag 23	9.7	0.194	0.131	1.49	0.47	DNA	50	27/11/2018 11:49:22 ص
25	post amp & defrag 24	101.7	2.034	1.098	1.85	1.66	DNA	50	27/11/2018 11:49:58 ص

Nanodrop readings for the purpose of the quantification of samples post-amplification and defragmentation and before the denaturation and hybridisation steps (120-fold mass dilutions)

APPENDIX X- Diversity metrics

Name	Units	Description
richness	OTUs	Number of OTUs with at least one read for the sample.
mirror	OTUs	See mirror estimator.
FE	OTUs	See singleton-free estimator.
chao1	OTUs	Chao-1 estimator, calculated as N + S^2 / (2 D) where N is the number of OTUs, S is the number of singleton OTUs and D is the number of doublet OTUs, i.e. OTUs with abundance 2.
shannon_2	bits	Shannon index (logs to base 2).
shannon_e	nats	Shannon index (logs to base e).
shannon_10	dits	Shannon index (logs to base 10).
jost	OTUs	Jost index of order q where q is specified by the -jostq command-line option, default 1.5.
jost1	OTUs	Jost index of order 1, the effective number of species given by the Shannon index.

Evenness metrics

Name	Units	Description
simpson	Probability	Simpson index, calculated as the sum over OTUs of f^2 where f is the frequency of the OTU. It is the probability that two randomly selected reads will belong to the same OTU. A value close to 1 indicates that a single large OTU dominates the sample, small values indicate that the reads are distributed over many OTUs.

dominance	Probability	Probability that two randomly selected reads will belong to different OTUs. Calculated as 1 - Simpson.
equitability	?	Also called Peilou's evenness. Entropy (Shannon index) divided by the logarithm of the number of OTUs. Value of 1 indicates perfectly even (equal abundances), small values indicate a highly skewed abundance distribution.
robbins	Frequency	Robbins index, calculated as S / (N + 1) where S is the number of singleton OTUs and N is the total number of OTUs.
berger_parker	Frequency	Berger-Parker index. Frequency of the most abundant OTU. A value close to 1 indicates that a single large OTU dominates the sample, small values indicate that the reads are distributed over many OTUs.

Other

Name	Units	Description
reads	Reads	Total number of reads for the sample.

Available from https://drive5.com/usearch/manual/alpha_metrics.html accessed 16/03/2019.

APPENDIX XI- Gant Chart

GHANT CHART showing the timelines (year 1)